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The Editor comments

IN RECOGNITION OF ACHIEVEMENT

During its long history the American Pharmaceutical Association has, in various ways, traditionally attempted to recognize and call attention to especially noteworthy contributions made by scientists in the fields of pharmaceutical research. As a primary example, for many years the Association has annually conferred two awards for specific phases of scientific contribution; one award for the most outstanding paper or series of papers published in its journals during the preceding year, and the other for significant research by an undergraduate pharmacy student.

With the tremendous growth and expansion of pharmaceutical research, which has been accompanied by a proportionate rise in high quality contributions, it was felt that the program of awards in the field of pharmacy could similarly be enlarged. Through the cooperation and generosity of seven major pharmaceutical manufacturing firms this goal has now been realized. The A. PH. A. Foundation—a separate agency which was established to manage such philanthropic activities—will administer these seven new awards.

We feel that this augmented award program is a most emphatic and conclusive demonstration of the fact that pharmacy continues to be aware of the important role played by that segment of the profession which is engaged in the pursuit of new knowledge through research. It is ample evidence, furthermore, that the Association feels that those individuals who make particularly outstanding contributions in this field should be singled out for special praise and honor by the entire profession. It is with this noble intent that the A. PH. A. Foundation Research Achievement Awards have been established.

Edward G. Feldmann

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Review Article

Biopharmaceutics: Absorption Aspects

By JOHN G. WAGNER

THERE APPEARS to be a rapidly growing interest in the effects that the dosage form of a given drug and the route of administration have on the biological effects elicited by the drug. This problem has been discussed at some length in the literature but is largely unrecognized by many in the biological and medical professions. Often it is assumed that the nature and intensity of the biological response obtained with a specific chemical compound in animals and man is due only to the inherent activity of the *molecular structure* of the compound. However, since dissolution, diffusion, absorption, transport, binding, distribution, adsorption on and transfer into cells, metabolism, and excretion are also intimately involved in drug action, the molecular structure, although vitally important, is only one factor in drug action. The term "dosage form" above includes the chemical nature (salt or simple derivative), physical state (amorphous or crystalline, solvated or nonsolvated, polymorphic form, etc.) and the particle size distribution and surface area of the drug itself in the dosage form.

The term *biopharmaceutics* was recently coined (1). In its broad sense we may define biopharmaceutics as the study of the relationship between some of the physical and chemical properties of the drug and its dosage forms and the biological effects observed following administration of the drug in its various dosage forms.

This broad definition would include drug latentiation (2) and the preparation of different salts of a given acidic or basic drug for the purpose of altering the biological effects elicited by the parent drug. Hence, biopharmaceutics encompasses the study of the relation between the nature and intensity of the biological effects observed in animals and man and the following factors: (*a*) simple chemical modification of drugs such as formation of esters, salts, and complexes; (*b*) modification of the physical state, particle size and/or surface area of the drug available to the absorption sites; (*c*) presence or absence of adjuvants in the dosage form with the drug; (*d*) the type of dosage form in which the drug is administered; and (*e*) the pharmaceutical process or processes by which the dosage form is manufactured.

Since biological screens in animals and man are often "one point determinations" with respect to time, the effect of the above factors are often not ascribed to biopharmaceutics but to the inherent activity of the molecular structure of the particular compound under investigation. Usually one must obtain the intensity of the biological response as a function of time to ascertain the effects of the factors outlined truly.

This review is circumscribed. It deals only with pharmaceutical and other factors which

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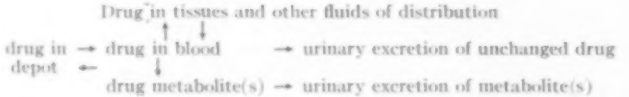
may have an effect on the rate and/or extent of absorption of drugs. The rate of absorption of a drug, and/or the percentage of the administered dose which is absorbed, can have a profound effect on the intensity of the biological response which is measured in animals and man. This is an area in which the industrial and practicing pharmacist, the pharmacologist, and the physician should have a good grasp of the fundamentals. The reviewer believes that it is in the area defined as biopharmaceutics above that a large part of the future of pharmacy lies.

James B. Conant, one of the great teachers and scientists of our age, wrote: "There must be constant critical appraisal of the progress of science and in particular of scientific concepts and operation." The reviewer was requested to write a critical review of the subject matter and he has attempted to do so. Perhaps some readers will disagree with the discussion and treatment in various sections. This is good. The purposes of the review are: (*a*) to outline some of the fundamentals of the absorption of drugs; (*b*) to indicate why absorption is often a problem in the administration of drugs by many routes; (*c*) to attempt to review some of the more recent and older literature concerning absorption of drugs; (*d*) to try to stimulate scientists to do research in this most important area of pharmaceutical and medical research.

FUNDAMENTAL PRINCIPLES

Blood and Tissue Levels—Biological Activity Relationships.—A simplified scheme for drug distribution was given by Teorell (3) as follows:


Drug in tissues and other fluids of distribution
drug in depot
drug in blood
urinary excretion of unchanged drug
drug metabolite(s)
urinary excretion of metabolite(s)

We may consider the depot, blood, urine etc., each as a separate compartment. When the drug, or a metabolite of the drug, moves from one compartment to another there are one or more rate constants acquainted with such passage. In most cases it has been shown that these are first-order rate constants. The above scheme is too simple for many drugs, but even this simple scheme has been adequate to explain the kinetics of absorption, distribution, excretion, and metabolism of many drugs and chemicals. If protein binding of the drug occurred in the blood then one or more constants would have to be added to the scheme (4).

The above scheme, or more complicated ones, indicate that for drugs which exert a pharmacologic effect there must be some relationship between blood and tissue levels and the biological activity exerted by the drug. Many in the biological and medical professions, who are not versed in kinetics, appear to feel that if there is not a direct proportional relationship then there is no relationship. Consideration of examples where the kinetics have been elucidated (5), of the models of Teorell (3), and of those shown in this manuscript indicate the relationships are not simple. In particular, consideration of kinetics indicate one would not usually expect the time after a single dose at which the peak blood level occurs to coincide with the time of peak biological activity. Often the peak biological activity will be delayed from the peak blood level with respect to time.

One should not only think in terms of biological effects after single doses of drugs but also these effects after multiple doses since there is usually accumulation of the drug in the body on most dosage regimens. The amount of drug in the body, A_b, at time t is the product of the concentration, C, of the drug in the fluids of distribution at time t and the volume of distribution, V_d. Hence, by definition, A_b does not include drug in the gastrointestinal tract, unchanged drug in the urine, or metabolized drug. After repetitive dosing, A_b depends upon the initial and maintenance doses, the rate of absorption the k_b of the drug, the dosage interval, Δt, and the number of doses, n, which have been administered. As n becomes very large, A_b becomes essentially independent of n (4, 6). The amount of drug at any particular site, where it acts, is related to A_b, at any time t, by a number of kinetic constants and time.

The usual nature of the biological activity (B_A)-amount of drug in the body (A_b) plot is shown in Fig. 1; this is a modification of one of the figures in the paper of Van Gemert and Duyff (7). The actual shape of the *S*-curve relating B_A and A_b will be different for different therapeutic agents. For example, if the distance between $(A_b)_{B_A = 0}$ and $(A_b)_{B_A = 1}$ approaches zero then the agent is said to have an all-or-none effect (7).

The usual response-dose plot is also *S*-shaped like the one in Fig. 1. This would be expected since such plots result from measuring response (or biological activity) after single doses of different magnitude, and under these circumstances

$$F \cdot \text{Dose} = k_b \cdot \int_{t=0}^{t=\infty} A_b \cdot dt = V_d \cdot k_b \cdot \int_{t=0}^{t=\infty} C \cdot dt \quad \text{(Eq. 1)}$$

where F is the fraction of the dose absorbed, k_b is the first-order rate constant representing loss of the drug from the body, and the integrals represent the area under a plot of A_b against time and the area under the plasma level-time plot after a single dose, respectively (8). It is obvious from Eq. 1 that A_b, at some time t after a given single dose, depends a great deal on the values of F and k_b. This fact is usually not well recognized when comparisons are made between different drugs by means of bioassays. To get closer to a true molecular comparison between two drugs which have the same type of activity, perhaps the dose ratios, based on Eq. 1, should be

$$\frac{(\text{dose})_1}{(\text{dose})_2} = \frac{F_2}{F_1} \cdot \frac{(MW)_1}{(MW)_2} \cdot \frac{(V_d)_1}{(V_d)_2} \cdot \frac{(t_{1/2})_2}{(t_{1/2})_1} \quad \text{(Eq. 2)}$$

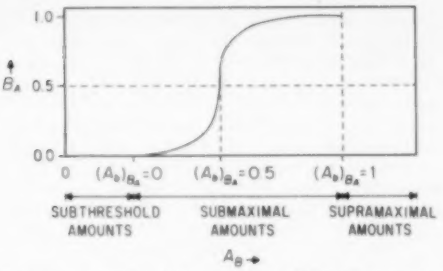


Fig. 1.—General shape of the biological activity (B_A)-body drug content (A_b) curve.

where the doses are in moles of compound, MW is the molecular weight, and $t_{1/2}$ is the biological half-life. The subscripts refer to compounds 1 and 2, respectively. In such a case the integrals, $\int_{t=0}^{t=\infty} A_b \cdot dt$, in terms of moles of compound, would be the same in each case and a true molecular comparison could be made. With one-point-in-time determinations of B_A, the rate of absorption of drug obtained with different dosage forms of the same drug, and different rates of absorption of drug obtained with the same type of dosage form with different drugs, can markedly affect results.

If the slope of the curve, dB_A/dA_b, at each A_b value in Fig. 1 were calculated and plotted against A_b, the resulting plot would be very similar to a blood level-time plot, i.e., like a bell-shaped curve which is skewed to the right. The area under the curve would be unity. The A_b value at which B_A is changing most rapidly, i.e., (dB_A/dA_b) = a maximum, would be approximately $(A_b)_{B_A} = 0.5$ in this example.

In 1937 Teorell wrote: "As it seems reasonable to assume that a great many pharmacological effects run closely parallel to the drug concentration in the blood, the conclusions to be drawn . . . in regard to such concentrations may directly apply to the magnitude of the effects produced." In these cases, the B_A *versus* t plot will be similar in shape to the blood level (or A_b) *versus* t plot. The area under the B_A, t plot is the total integrated activity of the single dose and should really be used for comparison purposes rather than the peak B_A values or, as in the usual case, just the value of B_A at some arbitrary time t. Some pharamacologists and clinicians use this more accurate estimate of activity. For example, Winter and Flataker (9) reported both the mean peak response and the mean total response in investigation of the effects of hormones upon the response of animals to analgesic drugs. The mean total response was the area under the mean reaction time-time after injection plots. Similarly, Houde *et al.* (10), in testing analgesic drugs in man, totaled the six hourly relief scores for each drug and obtained an estimate of the area under the mean pain relief-time after administration plot. They stated that it was these total relief scores which they employed for statistical analysis of drug effects.

In the cases cited above, where the B_A *versus* t plot is like a blood level plot, then one would expect that a plot of the logarithm of B_A *versus* t would yield a straight line past the maximum value of B_A. Swintosky and Sturtevant (11) published such plots for: (*a*) the pyretogenic effect of *d*-lysergic acid diethylamide following intravenous administration in rabbits, (*b*) the salivary response to tincture of belladonna after oral administration to man, and (*c*) the mydriatic effect of 9-methyl-3-oxo-9-azabicylo-[3,3,1]-nonan-7-yl-benzilate maleate administered orally to mice. These authors showed the utility of the pseudo first-order rate constants for the design of oral sustained action products. However, Nelson (12) published almost an identical mathematical treatment three years previously. The principal difference between the papers is that Nelson used the rate constant obtained from the blood level-time plot, and Swintosky and Sturtevant used the rate constant obtained from the biological activity-time plot.

Relating the intensity of the biological activity of a specific drug to the kinetics of its absorption, distribution, and metabolism is actually the most fundamental type of research one could perform with that drug. It is unfortunate that so few investigators in the medical and biological professions have done research in this area with specific drugs. Most of the methods and mathematics are available, and the rewards and results would be well worthwhile, both scientifically and economically.

A few references where investigators have elucidated some of the relationships between biological activity and blood and tissue levels will be cited. Foldes, *et al.* (13), stated: "Whatever the route of administration, in the final analysis, the toxicity of local anesthetic agents depends on their plasma concentrations." In considering the physical and physio-chemical factors affecting the action of muscle relaxants, Foldes (14) stated: "The intensity of action of muscle relaxants depends on their concentration at the neuromuscular junction. This, in turn, depends on the plasma level of the agent." An example involving a metabolite is Nirvanol (5-ethyl-5-phenyl hydantoin) a metabolite in the dog and man of Mesantoin (3-methyl-5-ethyl-5-phenyl hydantoin). Butler (15) reported that administration of Mesantoin, in the usual clinical dosage schedule, can be expected to result in larger amounts of Nirvanol in the body than of unchanged Mesantoin. He had no convincing evidence that the therapeutic effects of Mesantoin could not be accounted for on the basis of the Nirvanol which was produced.

In the above examples the drugs considered exert a specific type of pharmacologic action. The antibiotics, however, are administered to inhibit the growth or kill microorganisms in the bodies of patients. Eagle, *et al.* (16), reported that the rate at which bacteria die under the impact of penicillin *in vivo* is independent of its absolute concentration, provided only that the latter is in excess of the level which *in vitro* kills the particular organism at the maximal rate. Large doses of penicillin are more effective than small doses primarily because of the longer time during which they provide effective concentration. Knothe and Mahler (17) showed that, *in vitro*, high concentrations (6.25 to 25 mcg./ml.) of tetracycline acted bacteriocidally against a large number of strains of *Staph. aureus* and *Strep. faecalis* but low concentrations (0.78 to 6.0 mcg./ml.) had only a bacteriostatic action. They reported, however, that only intravenous administration of tetracycline is capable of providing concentrations in the bacteriocidal range in man.

An example where the biological effect is very

remote with respect to the time of administration, but has been adequately explained on a kinetic basis, is that of digitoxin. The work of Okita, *et al.* (18), makes excellent reading. By use of biosynthetically-labeled digitoxin, administered intravenously to patients with congestive heart failure, they showed that digitoxin has a very long residence time in the body. After autopsy of some patients they showed that only about 2 mcg. of digitoxin per 100 Gm. of ventrical tissue from the heart could be found and that this represented less than 1% of the administered dose. Swintosky (19) reported the half-life of digitoxin, calculated from twenty-day urinary excretion data to be 5.3 days, but, unfortunately, did not relate it to the work of Okita, *et al.* Okita also found that the myocardium has no special affinity for digitoxin in comparison with other organs and that the amount there at any time after administration is explicable on the basis of the kinetics and distribution. Another interesting aspect of this work was proof that biliary excretion of unchanged digitoxin is followed by reabsorption in the small intestine. Hence, the drug keeps cycling back and forth between the vascular system and the intestine.

Kinetics of Absorption.—The rate of absorption and the extent of absorption (i. e., the per cent of the dose absorbed) are very important factors in determining the magnitude of the blood and tissue levels with respect to time after administration and, hence, in determining the intensity of biological activity. Nelson (20) and Dominguez (5) have published excellent reviews on the kinetics of absorption, distribution, and excretion. This review will only contain sufficient material on this subject to make other parts of the review more intelligible.

Except for an intravenous injection in which the rate of injection is experimentally controlled, in all other administrations a drug enters the blood stream at an unknown rate. Under certain circumstances this rate can be determined. Dominguez (5), Nelson (21), Swintosky (22), and others have shown that many substances administered orally exhibit a steady state of diffusion during absorption, first-order metabolic conversion, and/or first-order urinary excretion of unchanged drug and metabolite(s). If these fundamental premises are fulfilled by a given drug then one can calculate the instantaneous rate of absorption at different times after oral administration by at least three known methods. The first method, that of Dominguez (5), requires a knowledge of the volume of distribution of the drug. The method is based on application of Eq. 3:

$$R = V_1 \cdot \frac{dC}{dt} + (A + B)C \qquad \text{(Eq. 3)}$$

where R = the instantaneous rate of absorption at time t in mg./hour; V_1 = the reduced volume of distribution and $V_1 = A/k_2$ where V_1 is in ml. and k_2 in hr.$^{-1}$, dC/dt = the instantaneous slope of the blood level curve at time t; C = the plasma concentration in mg./ml.; A = the renal clearance with dimensions ml./hr. A is the slope of the straight line when rate of excretion of unchanged drug in the urine (mg./hr.) is plotted against plasma concentration, C, in mg./ml. B = the slope of the straight line when rate of metabolic conversion of the drug (mg./hr.) is plotted against C in mg./ml. The dimensions are ml./hr. $B = V_1 \cdot k_1$ where k_1 is the first-order rate constant (in hr.$^{-1}$) for metabolic conversion of the drug.

From the above we may also write

$$A + B = V_1(k_1 + k_2) = V_1 \cdot k_b \qquad \text{(Eq. 4)}$$

where k_b is the first-order rate constant for loss of drug from the blood. If there is no metabolic conversion of the drug then the third term of Eq. 3 reduces to $V_1 \cdot k_2 \cdot C$. If there is metabolic conversion of the drug, the constant B cannot be evaluated from the unchanged drug recovered in the urine because of uncertainty about the complete absorption of the drug. Independent experiments where the drug is administered intravenously, however, allow computation of the constant B(5).

The second method of calculating instantaneous rates of absorption is that used by Nelson (21) and others. This method involves application of equation (5):

$$R = \frac{1}{f}\left(\frac{1}{k_b} \cdot \frac{d^2Ae}{dt^2} + \frac{dAe}{dt}\right) \qquad \text{(Eq. 5)}$$

where R = the instantaneous rate of absorption at time t in mg./hr, f = the fraction of the drug absorbed which is excreted unchanged in the urine; k_b has the significance indicated above; d^2Ae/dt^2 = the second derivative of a plot of cumulative amount (mg.) of unchanged drug excreted in the urine against time in hours; dAe/dt = the first derivative of the same plot.

The third method, that of Dominguez (5), involves the assumption that the blood concentration curve, C *versus* t, can be fitted with a three-term exponential equation of the type

$$C = a \exp \cdot -\alpha t - c \exp \cdot -\gamma t + d \exp \cdot -ft \qquad \text{(Eq. 6)}$$

Evaluation of the constants as he indicates allows plotting of the rate of absorption (mg./hr.) as a function of time and, hence, also of cumulative amount absorbed as a function of time. Equation 6 assumes that the rate of absorption, R, involves the two exponential terms as follows

$$R = P(\exp \cdot -\gamma t - \exp \cdot -ft) \qquad \text{(Eq. 7)}$$

where

$$R = \frac{AC(\gamma - \alpha)}{\alpha} = \frac{Ad(f - \alpha)}{\alpha}$$

Now Teorell (3) and others, including Krüger-Thiemer (4), assumed that the rate of absorption could be represented by a single exponential function leading to an equation of the type

$$C = C_0 \cdot \frac{k}{k - k_b} \cdot (\exp \cdot -k_b t - \exp \cdot -kt) \qquad \text{(Eq. 8)}$$

where k is the first-order rate constant for the absorption process, C_0 is the amount absorbed divided by the volume of distribution, and the other symbols have the significance indicated above. In his article, Krüger-Thiemer (4) stated that k may be gained from Eq. 8 by series evolution for $\exp.^{-(k - k_b)t}$, whereby three approximations may be calculated. He gave the first and second of these approximations. The difficulty with application of this method, as Dominguez (5) pointed out, is that Eq. 8 leads to the conclusion that the rate of absorption jumps instantaneously from zero to its largest value either at the time of administration or after some lag

time. In reality, for cases which have been investigated the plasma level-time plot obtained following oral administration had four points of inflection. The first inflection point occurs shortly after administration, usually within the first one-half to one hour. The second point of inflection corresponds to the time when the rate of absorption is a maximum. The third point corresponds to the peak plasma level at which time the instantaneous rate of absorption is exactly equal to the instantaneous rate of excretion. After the maximum plasma level there is a fourth point of inflection corresponding to the time absorption has ceased and the plasma level-time plot becomes a simple exponential function of time, namely $C = C_0 \cdot \exp.^{-kt}$. Hence, application of Eq. 8 to obtain the rate of absorption may lead to large errors. However, the equation is very useful as a simple model to represent an approximate blood level-time plot. A factor which was discussed by Nelson (20) is the effect of stomach emptying rate on the nature of the absorption curve. Equations 7 and 8 really describe the amount of substance in compartment C as a function of time for two consecutive first-order reactions namely

$$A \xrightarrow[(\text{or } k)]{f} B \xrightarrow[(\text{or } k_b)]{\gamma} C \qquad \text{(Eq. 9)}$$

It is conceivable that the $A \rightarrow B$ reaction may be stomach emptying which has been shown to obey first-order kinetics in many cases (see Gastrointestinal Physiology section).

There are many problems connected with the application of these methods. The first method necessitates determination of the volume of distribution of the drug which in many cases is subject to considerable error. The second method requires no knowledge of the volume of distribution, but there is some doubt in the reviewer's mind that the derivatives, dA_e/dt and d^2Ae/dt^2, can be obtained with considerable accuracy unless a large number of points are available for the excretion curve in the region where the derivatives are determined. Particularly where the drug has a long half-life (> ca. six hours) and the dosage form being studied allows slow absorption of the drug, one may interpret a certain segment of a plot of cumulative amount of drug excreted against time as a straight line when in reality the line is curved and has an inflection point in this region. The line may be only *apparently* linear because the number of points are too small upon which to make a sound statistical decision. Some of the model diagrams, Figure 2, illustrate this point well. Perhaps the difference is only of academic interest in one sense but in another sense it may be quite important. For example, one could conclude, on insufficient evidence, that a given dosage form was releasing the drug at a constant, zero order rate, when in reality it may be releasing the drug at a slow first order rate. This could be true, for example, in the interpretation of the kinetics of excretion of amphetamine from product F in the paper of Campbell *et al.* (23). The third method involves assignment of two of the constants, namely γ and f, to the absorption process. This method has been shown by Dominguez (5) to be applicable to the absorption of creatinine in the dog and man. Such equations as Equation 6 are useful to prepare models to illustrate the relative effects of change in "absorption rate," k_b, and other factors would have on plots of amount of drug in the depot *versus* time, amount of drug in the body *versus* time, and cumulative amount excreted *versus* time. The models shown in Figure 2 illustrate this. The models are similar, but different in some aspects, to those of Teorell (3), De Jongh and Wijnans (24), Van Gemert and Duyff (7), Krüger-Thiemer (4), and Garrett, *et al.* (see footnote).[1]

Hypothetical Models of the Kinetics of Absorption and Excretion Following Administration of Slowly Releasing Dosage Forms.—The models (Fig. 2 and Table I)[2] were designed to show the relative effects of stomach emptying and slow release of drug, by either a zero-order or first-order process, on a resulting hypothetical "blood level," if the drug is removed from the blood by a first-order process. In all cases, the rate constant, k_b, for removal of drug from the blood (and other fluids of distribution) is taken as 0.1155 hr.$^{-1}$, which corresponds to a half-life of six hours; this is the average value for salicylic acid in human subjects. In all cases, curve C would correspond to the "blood level," or actually, the fraction of the total drug in the fluids of distribution. Curve D corresponds to the fraction of the total drug which has been excreted and/or metabolized to time t. If the drug was not metabolized, but excreted unchanged in the urine, then curve D, in each case, would correspond to the cumulative amount of the drug in the urine to time t. Curves A and B correspond to the amounts of drug in compartments A and B, respectively. For example, in model I, curve B corresponds to the amount of slowly available drug still present in the dosage form at any time t. The constant, k_1, for release of drug in quickly available form in model I, and for stomach emptying in models II and III, was chosen as follows: when $k_1 = 1.0$ hr.$^{-1}$ it requires 2.772 hr. (four half-lives) to release or empty 93.75% of the original material. Since $X_A = 1$ at $t = 0$ in both models II and III, a k_b of 0.2 hr.$^{-1}$ in model II corresponds approximately to a k_2 of 0.5 hr.$^{-1}$ in model III on the basis that after about five hours essentially all the $B \rightarrow C$ process, if considered alone, would be complete; similarly a k_b of 0.05 hr.$^{-1}$ in model II corresponds approximately to a k_2 of 0.1 hr.$^{-1}$ in model III on the basis that after about twenty hours essentially all the $B \rightarrow C$ process, if considered alone, would be complete.

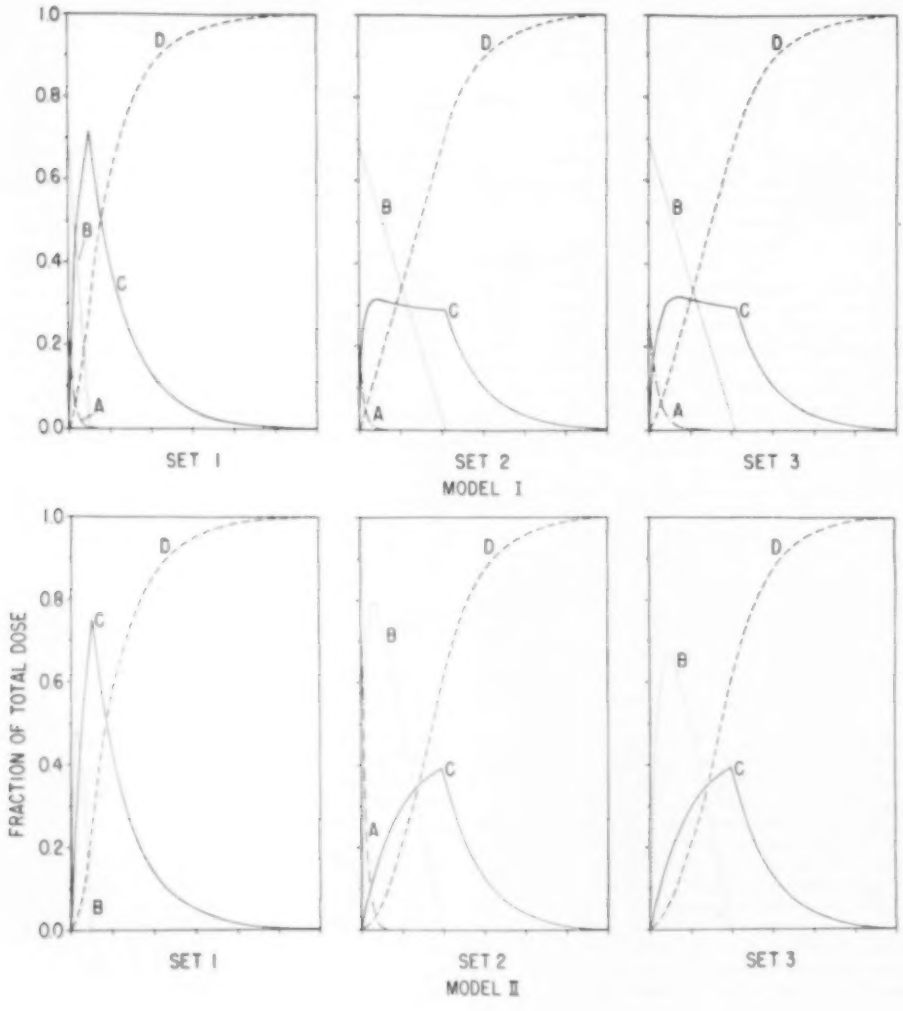
Careful study of the plots in Fig. 2 (see Appendix also) shows the effect of changing one of the rate constants while the other two are held constant. One can also visualize why tissue levels of drug, or level of drug at the site of action, would not usually correspond with the blood level in relation to time since the amount of drug in such tissues would involve one or more compartments and rate constants leading off from the C compartment. Hence, in the usual case, one would not expect peak biological action to correspond with peak blood level in rela-

[1] The plots shown in Fig. 2 were kindly provided by Dr. E. R. Garrett and C. D. Alway of the Department of Physical and Analytical Chemistry of The Upjohn Co. The analog computer used was described by Garrett, *et al.* [*J. Pharm. Expti. Therap.*, **130**, 106(1960)]. The programming of the analog computer for the plots shown in Fig. 2 will be described in a future article entitled: "Pharmaceutical Application of the Analog Computer I."

[2] The differential equations and their solutions, shown in Table I, were kindly provided by Dr. J. I. Northam of the Statistical Section of The Upjohn Co.

tion to time. The plots help one to visualize the differences one obtains when a drug is given in a quickly available form and in a prolonged action or slowly releasing form. This can be seen, for example, by comparing curves A and B in model I.

The Relationships Between Dosage Forms, Optimal Dosage Schedules, and the Kinetics of Absorption.—Most oral dosage forms may be broadly divided into three types based upon the rapidity with which the drug is absorbed after adminstration. The first type would be those which, when administered, allow extremely rapid absorption of their contained drug. Many solutions, some suspensions containing the drug in very small particle size, and some ordinary compressed tablets, which rapidly disintegrate and liberate the drug in very small particle size, could be included in this group. The second type encompasses the true sustained release or prolonged action dosage forms. A properly designed dosage form of this type produces after a single dose an essentially constant A_b, which is above that level needed for suitable therapeutic activity but below the peak level obtained with the same dose of drug in a quickly available dosage form, for a period of time which is some multiple of the time that the quickly available dosage form would maintain a level of drug above the level needed for therapeutic activity. The principle, upon which most dosage forms of the latter type is based, is slowing of the absorption rate of the drug by slowing the rate at which the drug is released to the absorption sites in the gastrointestinal tract. Properly designed and functioning, a sustained release or prolonged action dosage form administered orally acts, during some time interval following administration, like a continuous, constant rate infusion or a constant surface pellet implant. Some commercial products, which are marketed as sustained release or



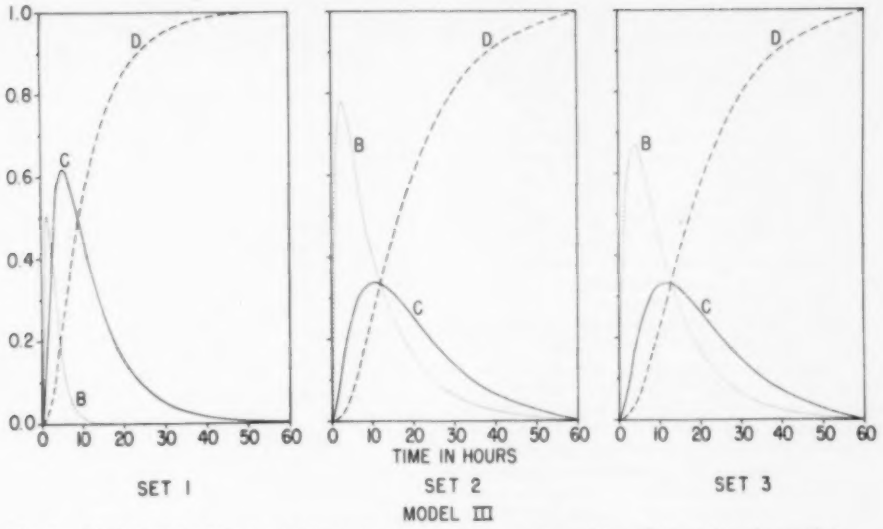


Fig. 2.—Models of the kinetics of absorption, distribution, and excretion of drugs (see Appendix).

TABLE I.—CONCENTRATION OF HYDROGEN ION AND HYDROXIDE ION AT VARIOUS pH VALUES

pH	Concentration of Ions in Microequivalents[a]/Liter (Micronormality) at 24°	
	Hydrogen Ion	Hydroxide Ion
1	100,000	0.0000001
2	10,000	0.000001
3	1,000	0.00001
4	100	0.0001
5	10	0.001
6	1	0.01
7	0.1	0.1
8	0.01	1.0

[a] Microequivalent = 10^{-6} equivalent weight.

prolonged action forms, perform their intended function while others apparently do not (23, 25, 26). Many, and probably most, oral dosage forms fall into a class intermediate between the above two types. A dosage form of the third type, we may say arbitrarily, acts in such a manner that the drug contained in it enters the blood stream at instantaneous rates which are changing quite rapidly over a period of time from one to several hours after administration. The net result, following oral administration of dosage forms in this group, is that the maximum amount of drug in the body from a specific single dose occurs at some time greater than one hour after administration and there are large variations in A_b at different times between the time of administration and the time when the drug has, for all practical purposes, disappeared from the body.

Another important field in therapeutics encompasses the choice of the best type of dosage form and the best type of dosage schedules for specific drugs. A few far-sighted investigators (3, 4, 7, 24, 27) have attempted, using a mathematical approach, to formulate some general rules which may serve as a foundation for practical work in this field of investigation. Van Gemert and Duyff (7) stress the following: "As to medical practice, it is strongly suggested that the elimination rate (i.e., determination of k_b) of important drugs be studied; this alone would be a great help toward rational treatment." This statement, published ten years ago, appears to have been lost in the literature and unappreciated by the large majority of clinical investigators and pharmacologists. Once the k_b of a specific drug is known, then a rational approach to the calculation of optimal dosage schedules and choosing of suitable dosage forms can be made. Also, many of the equations derived or reviewed by the above investigators may be applied to any drug. Some of the practical consequences of the calculations will now be considered.

Van Gemert and Duyff (7) concluded the following: (a) If a drug is administered in a single dose which is not, or very infrequently, repeated then a dose approximately equal to 2.5 times the dose giving a half-maximal effect [i. e., dose = $2.5\,(A_b)_{B_A\,=\,0.5}$] is the most economical way of obtaining the desired biological activity, B_A. (b) If a drug is repetitively administered then the economically favorable dose per unit of time, and the way in which it should be divided into individual doses at the appropriate intervals, depend on the general shape of the B_A *versus* A_b plot of the drug in question. In general, they concluded, for low overall dosages the individual doses can be fairly large, and correspondingly widely spaced. For high overall dosages, however, treatment becomes, generally speaking, the more economical as the permanent infusion is more nearly approximated. As pointed out formerly, a properly designed and functioning sustained-released or prolonged action dosage form and a constant surface pellet implant are very similar to a permanent infusion. Van Gemert and Duyff (7) pointed out that these general rules apply not only to substances artificially introduced, but also to drugs produced by the body itself. Hence, one could conclude these general rules apply to drugs which

simulate endogenous active compounds or to drugs which are converted to a compound which also exists endogenously and is biologically active.

Similarly, De Jongh and Wijnans (24) from their mathematical analysis stated: "Economy is increased on using a small dose which is frequently repeated. Repetition of a dose, however small, at the right moment, is always more profitable if it is only in equilibrium with the speed of elimination. By permissible extrapolation the great economy of the body on implantation of a crystalline tablet is explained: there is then in a manner of speaking a continuous infusion which may be compared with an infinitely often repeated infinitely small dose, with an infinitely small time interval."

Boxer, *et al.* (6), derived equations for the accumulation of a drug given in several doses, D, at constant intervals, Δt, assuming the drug was administered by rapid intravenous infusion (i.e., all the dose instantly in the volume of distribution). In such a case, when the logarithm of the amount of drug in the body (A_b) is plotted against time one obtains a series of "saw-tooths" extending upwards until at some time the median curve flattens out and the value of A_b from that time on will fluctuate back and forth between two extreme levels. The equations for the two extreme levels after a large number of doses approach

$$A_{b_\infty} = \frac{D}{1 - \exp{\cdot -k_b \Delta t}} \qquad \text{(Eq. 10)}$$

$$A_{b_{\infty-1}} = \frac{D \exp{\cdot -k_b \Delta t}}{1 - \exp{\cdot -k_b \Delta t}} \qquad \text{(Eq. 11)}$$

where A_{b_∞} is the upper limiting value of A_b at the start of the interval Δt, $A_{b_{\infty-1}}$ is the lower limiting value of A_b at the end of the interval Δt, and D is the dose administered.

It is difficult to apply the equations of Boxer, *et al.* (6), to calculate the results after oral administration unless one has some knowledge of the rapidity of absorption from the dosage form administered. If absorption is very rapid compared to the dosage interval, Δt, then the equations give a good estimate of the equilibrium situation (4, 28). However, the slower the absorption process, the less accurate are Eqs. 10 and 11. For a sustained action preparation, $\Delta t \to 0$, $\exp{\cdot -k_b \Delta t} \to 1$ and the denominators of Eqs. 10 and 11 approach zero, hence, the ratios would approach infinity for Eqs. 10 and 11. It is obvious one must be careful where these equations are applied.

Solubility, Particle Size, and Physical Form.—The initial rate of dissolution, i.e., the rate at very low per cent saturations of the dissolution medium, is directly proportional to the solubility of the drug in the dissolution medium as it exists at the solid-side of the diffusion layer. In the case of gastrointestinal absorption of drugs, the dissolution medium is the gastrointestinal content at the particular physical location of the drug particle in the tract. If the dissolution process is diffusion-controlled, then the liquid in the diffusion layer right at the interface of the solid drug particle is essentially a saturated solution of the compound in the dissolution medium except for ions and molecules depleted in any reaction occurring in the diffusion layer (29). Hence, the rate at which molecules of the drug reach the bulk gastrointestinal content is determined in great part by the solubility of the drug as defined above. Other factors influence the rate of dissolution of a drug but these will be discussed in another section.

Both the absolute particle size, and the particle size and particle size distribution of a drug sample as it relates to surface area, may be important in the rate of dissolution of a drug (30). When the particle size is greater than about 10 μ, the rate of dissolution is directly proportional to the surface area. Hence here, surface area, and not particle size *per se*, is a factor controlling dissolution rate. However, when particles below 10 μ in diameter are considered, the effective particle radius and not the surface area may be more important (30).

Finely divided particles dissolve at a greater rate and have higher solubilities than similar macro particles (31–34). The only energy difference between the large and small crystalline forms is the surface energy (32). Increase of solubility due to reduction in particle size only becomes significant (beyond *ca.* 1%) for very small particles in the submicron range (32). Alexander (35) reported that the solubility of amorphous silica in water is related exponentially to the surface area of the silica. Hasegawa and Nagai (34) published a mathematical approach to the tendency for small particles to dissolve faster than larger particles.

If a compound is not very soluble it is sometimes dangerous to reduce the particle size to an extremely small range. For example, Desai, *et al.* (36), reported that rats fed aerated silica gel for prolonged periods developed abnormal large nodules in their intestines. Similarly, Sasson (37) found barium sulfate crystals in the lumen of intestinal glands following barium enemas; he said this may explain the portal of entry and mechanism of formation of barium granulomas of the rectum occurring after barium enemas. Inhalation of extremely finely divided compounds can be dangerous also. A commercial sample of amorphous silica, with a particle size of *ca.* 200 Å., a B.E.T. surface area of 145,000 cm.^{2}/Gm., and a water solubility of 41 mg./L. at 20°., caused more than doubling of the lung weights of albino rabbits which were exposed to an aerosol of the silica for eight hours each day for periods up to twenty-seven months (38).

There have been reports (39–42) that a given range of particle sizes of a drug produced a biological effect whereas larger particles produced little or no effect. In such cases it is most probably true that the compound's absorption is rate-limited by surface area. If the particle size is greater than a certain range, the range depending upon the compound, then insufficient compound is absorbed to produce an observable biological effect. This is obvious from the equations presented below in the section entitled Dissolution Rate.

When particle size is discussed it is often difficult to get a mental picture of the size range. The size distribution of tobacco smoke particles from cigarettes is in the range 0 to about 1 μ. The biggest peak on the distribution diagram occurs in the particle diameter range 0 to 0.14 μ (43). An extremely fine micronized powder will have a particle size distribution, as measured by Coulter counter, of from about 0.5 to 10 μ, and a surface area in the range about 6 to 12 $\times$ 10^4 cm.^{2}/Gm. A milled powder will have a particle size in the range of about

10 to 150 μ and a surface area of the order of 0.4 to 6 × 10^3 cm.²/Gm. Since most drugs are administered in doses below 0.25 Gm. it is obvious that the total available surface area of doses of crystalline substances as received from the chemist, or just milled, will be relatively small. The specific surface, in cm.²/cm.³, is equal to 6/d for spheres, and very near this value for truncated rods such as one finds usually in a micronized powder. Hence, a powder with a mean particle diameter of 10 μ will have about ten times the specific surface of the same compound with a mean particle diameter of 100 μ.

Metastable polymorphs have higher solubilities than the more stable forms of the same compound (32, 44). Hence, everything else being equal, one could deduce that the more soluble polymorphs would dissolve at faster rates and be more rapidly absorbed. An effect that is not often recognized is that dry grinding can cause phase transitions in solids. Cleverley and Williams (45) reported that grinding of crystalline barbituric acid derivatives often produce a change in polymorphic form. Similarly, Yamaguchi and Sakamoto (46) found that dry grinding of gibbsite caused ultimate decomposition of χ-alumina. Palen (47) reported that certain polymorphic forms of chloramphenical were inert biologically, and that the α-form of yellow iron oxide is biologically active yet does not affect ascorbic acid as much as other forms of iron oxide. Hydrates and other solvates of a given compound have different solubilities and rates of dissolution than the nonsolvated form (44). Hence conversion of a nonsolvated compound to a solvated compound during, or subsequent to, formulation of a product may cause difficulties or produce an effect which is unexpected. Such transitions are temperature-dependent. Sometimes only a complete study, where the logarithm of the solubility of the compound is plotted against the reciprocal of the absolute temperature, can clearly delineate the problem (44).

The equation of Dyer (48) relating the effect of pH on solubilization of weak acids and bases by surfactants which form micelles may be of interest not only from the standpoint of formulation, but also from the standpoint of oral absorption from solutions containing such agents. The equation reported by Krishnamurti and Mirza (49) relates the amount of substance adsorbed by an adsorbent to its water solubility. Their results agree with Freundlich's old statement: "The strongly adsorbable substances are for the most part difficultly soluble in water: the weakly adsorbable inorganic salts, acids, and bases are easily soluble."

Dissolution Rate.—Fick (50) put diffusion on a quantitative basis by adopting the mathematical equation of heat conduction derived some years earlier by Fourier. One way of expressing Fick's law is as follows: the quantity of solute, dW, which diffuses, at constant temperature, through an area, A, in a time, dt, when the concentration changes by an amount, dc, through a distance, dx, at right angles to the plane A, is given by the expression

$$\frac{dW}{dt} = -DA\frac{dc}{dx} \qquad \text{(Eq. 12)}$$

Hence the diffusion coefficient, D, is the amount of solute which will cross 1 cm.² of cross section in unit time if the change in concentration per cm. in a direction perpendicular to this cross section is unity. This law assumes that the only motion involved is due to molecular agitation and was proved by Fick for diffusion in one direction only, upward against the force of gravity.

The Noyes-Whitney law formulated in 1897 (51) concerns the rate at which solids dissolve in their own solutions when the surface area of exposed solid changes negligibly. This law states that the rate of concentration change, dC/dt, is at any instant directly proportional to the difference between the concentration of a saturated solution, C_s, and the concentration, C, existing in the solution at this instant.

$$dC/dt = k(C_s - C) \qquad \text{(Eq. 13)}$$

where k is a constant with dimensions 1/time. Integration with $c = 0$ at $t = 0$ yields

$$ln(C_s - C) = ln\ C_s - kt \qquad \text{(Eq. 14)}$$

or

$$C = C_s(1 - \exp\cdot^{-kt}) \qquad \text{(Eq. 15)}$$

Noyes-Whitney explained the dissolution process on the assumption that a very thin layer of saturated solution was formed at the surface of the solid and that the rate at which the solid dissolved was governed by the rate of diffusion from this saturated layer into the main body of the solution.

A few years later, Brünner and Tolloczko (52) showed that the value of the constant k (in Eqs. 13, 14, and 15) depended upon the surface area, S, of solid exposed, the intensity of agitation or velocity of fluid across the solid, the temperature, the structure of the surface, and the experimental apparatus.

Nernst and Brünner, who worked in Nernst's laboratory, advanced (53) a theoretical generalization of the law to include all kinds of heterogeneous reactions. They postulated that the velocity of a heterogeneous reaction was determined by the velocities of the diffusion processes that accompanied it. This included the concept that the equilibrium is set up at the boundary surface practically instantaneously compared with the rate of diffusion. According to Davion (54), these considerations led to the following equation which, as a first approximation, describes the dissolution of a solid by a diffusion controlled process.

$$dW/dt = D/h \cdot S \cdot (C_s - C) \qquad \text{(Eq. 16)}$$

where h is the thickness of the diffusion layer and the other symbols have the meanings above. This explains the variation of the constant k with intensity of agitation since the more intense the agitation, the thinner the diffusion layer, and hence the greater will be the rate of dissolution, dW/dt.

Wilderman (55) and Zdanovskii (56) subjected the entire Nernst-Brünner diffusion theory to severe analysis and experimental study. Wilderman doubted that it explained the dissolution process. Zdanovskii, after experimentation with the dissolution of inorganic salts, claimed that he derived the following generalized equation. When the rate-determining step is the surface or interface process, the rate of dissolution is given by

$$dW/dt = \alpha S(C_s - C_y) \qquad \text{(Eq. 17)}$$

When the process is diffusion controlled, the rate of dissolution is given by

$$dW/dt = (D/h)S(C_g - C_s) \quad \text{(Eq. 18)}$$

For the general case the equation is

$$\frac{dW}{dt} = \left(\frac{\alpha D}{D + \alpha h}\right) S(C_s - C_x) \quad \text{(Eq. 19)}$$

where α is the rate constant of the interface process, C_s is the concentration at saturation, C_x is the concentration in the bulk solution, and C_g is the concentration in the boundary diffusion layer (presumably at the solid side). The other symbols have the significance as defined above. The rate constant, K, in the latter equation is given by:

$$\frac{1}{K} = \frac{1}{\alpha} + \frac{h}{D} \quad \text{(Eq. 20)}$$

In 1931 Hixson and Crowell (57) wrote an excellent review of the theory of the dissolution of solids and derived a new law (the cube root law) in which the velocity of solution of a solid in a liquid is expressed as a function of the surface area and the concentration. They claimed that the Noyes-Whitney law in its original form, without any assumptions regarding the mechanism by which the process takes place, has been quite generally substantiated by experiment. For their derivations and suggested applications the original paper should be consulted. The equations of Hixson-Crowell have been used by Wurster, *et al.* (58, 59), in their studies.

King and Brodie (60) and Hixson and Barm (61) studied the dissolution rates of benzoic acid in dilute aqueous sodium and potassium hydroxides. They explained their data on the basis of the Nernst-Brünner two film model of diffusion controlled kinetics. This theory assumes linear concentration gradients of all species in the diffusion layer. W. I. Higuchi, *et al.* (29), showed where this theory would be expected to fail. Using the Nernst-Brünner model and assuming nonlinear concentration gradients in a single diffusion layer, these authors derived equations which satisfactorily described the the rate of dissolution of weak acids and bases in various solutions and buffers. They showed that the complex rate equations could be reduced to more simple equations under certain conditions which may be readily determined by a consideration of the dissociation constants of the acids and bases involved.

The long duration of action of zoxazolamine, a centrally acting muscle relaxant, was ascribed by Brodie and Hogben (62) to the precipitation of the drug in the intestines, and, due to the low solubility of the drug in the gut, it is slowly absorbed during a period of many hours after oral administration. Juncher and Raaschou (63) ascribed differences in blood levels of penicillin V, obtained after administration of different salts and the free acid of this penicillin, to differences in solubility of the salts. The *in vitro* experiments, which the latter authors performed, were really experiments in which the rates of dissolution of the different salts and the free acid were compared, and not their solubilities. The order of peak blood levels of penicillin V obtained in human subjects was the same as the order of the rates of dissolution of the different forms *in vitro* as their data show. The faster the salt dissolved *in vitro* at pH 2 or 4, the higher was the average peak plasma level. In this case, as in some others, the order of the rates of dissolution of such a series of salts of a weak acid and the parent weak acid, the order of peak blood levels attained, and the order of the water solubilities of the drug are the same. However, one should not lose sight of the fact that it is really the relative rates of dissolution both *in vitro* and *in vivo* which is the important factor and such rates can be influenced by other factors than just solubility. This was pointed out by Nelson (64) who stated there was a poor correlation between the water solubilities and the solution rates of a series of theophylline salts.

To the reviewer's knowledge, Nelson (64) was the first to show that, with other factors remaining constant, the dissolution rates of a series of salts of a given weak acid determines the rate of build up of blood level with time and the maximum blood level attained. Earlier, Edwards (65) postulated that the dissolution of an aspirin tablet in the stomach and intestine would be the rate process controlling the absorption of aspirin into the blood stream. In later publications, Nelson (66) showed that: (*a*) dissolution rate was rate-determining in absorption of various tetracycline preparations providing initial surface area was restricted in the dosage form, (*b*) the absorption of aspirin appeared to be rate-limited by the time necessary for the drug to dissolve after ingestion as Edwards had predicted, and (*c*) the absorption of benzylpenicillin was rate-limited by the intrinsic dissolution rate properties of the potassium and procaine salts used.

A technique for studying dissolution rates of drugs, which does not require an assay method for the dissolving specie, but is based on weight loss of a constant surface pellet, was reported by Nelson (67). Using this method he showed that the sodium salts of several weakly acidic drugs dissolved much more rapidly than the corresponding weak acids in the pH range 1.5 to 9.0. Similarly he showed that admixture of tribasic sodium phosphate with a weakly acidic drug increased the rate of dissolution of all the acids studied. In all cases a maximum occurred in a plot of initial rate of dissolution *versus* fraction of acid in the mixutre. The location of the maximum appeared to be related to the dissociation constant of the acidic drug. Also, the maximum initial rates obtained with the mixtures were lower than those obtained with the salts of the acid used.

Equation 19 above may be written as

$$dW/dt = K \cdot S \cdot (C_s - C) \quad \text{(Eq. 21)}$$

where W is the amount dissolved, t is the time, K is the rate constant with dimensions distance/time, S is the surface area, C_s is concentration of dissolving solid at the solid side of the diffusion layer, and C is the concentration in the dissolution medium. At very low per cent saturations of dissolving substance in the dissolution medium, Nelson (64) showed that this reduces to

$$dW/dt = KSC_s \quad \text{(Eq. 22)}$$

which upon integration, with S considered a constant, yields

$$W = KSC_s t \quad \text{(Eq. 23)}$$

He pointed out that this should describe the *in vivo* dissolution process from a constant surface dosage form too, since absorption from solution following

dissolution would help keep C small in relation to C_s. From *in vitro* data a plot of W/S against t should yield a straight line with slope, KC_s, having the dimensions mg./cm.²/hr. if W is in mg., S in cm.², and t in hours. KC_s is the initial rate of dissolution and is the rate used by Nelson (64, 66, 67) and by W. I. Higuchi, *et al.* (29), in their plots. Hence, as stated before, the initial rate of dissolution is directly proportional to the solubility of the drug in the dissolution medium as it exists on the solid side of the diffusion layer.

By working at pH values above those where there are significant amounts of protonated form, Nelson (64) treated theophylline as a monobasic acid. He obtained a linear plot by plotting the initial rate of dissolution per unit surface area, i.e., KC_s, against $1+K_a/[H^+]_d$. Here K_a was the acid dissociation constant of theophylline and $[H^+]_d$ was obtained by measuring the pH of a saturated solution obtained by adding theophylline to the buffer or dissolution medium at constant temperature. The $[H^+]$ in such a saturated solution was considered to be the diffusion layer pH. If the profiles hypothesized by W. I. Higuchi, *et al.* (29), are correct then no definite pH can, apparently, be assigned to the diffusion layer. The pH will vary with the exact position in the film. Certainly the pH of the diffusion layer which Nelson describes is probably that pH right at the interface of the solid and the diffusion layer. Hence this is really a problem in semantics.

As Nelson (64) has shown, the total solubility, C_s, of a weak acid, HA, is given by:

$$C_s = [HA] + [A^-] = [HA] + [HA] \cdot \frac{K_a}{[H^+]} = [HA]\left(1 + \frac{K_a}{[H^+]}\right) \quad \text{(Eq. 24)}$$

where [HA] is the intrinsic solubility of the nonionized form of the acid, usually determined at very low pH, $[A^-]$ is the concentration of ionized acid, $[H^+]$ is the hydrogen ion concentration of the saturated solution, and K_a is the acid dissociation constant.

GASTROINTESTINAL ABSORPTION

Gastrointestinal Physiology.—A recent review by Hogben (68) on the alimentary tract emphasizes the physiology of absorption and secretion. It is pertinent in this review to discuss those factors in gastrointestinal physiology which may be important in the absorption of drugs and in the mechanism of action of certain types of dosage forms.

pH.—In man the stomach contents are usually in the pH range 1 to 3.5 and pH 1 to 2.5 is the most common range (69–76). The pH range 3.5 to 1 corresponds to from 320 to 100,000 microequivalents of hydrogen ion per L., respectively. pH is not a vivid way of expressing the high acidities reached in the stomach. This is clarified by a study of Table I. It is easy during the stress and strain of a laboratory day to forget what the pH scale really means. As an example, one should not average pH values but convert to a_{H^+} by the relationship, $a_{H^+} = 10^{-pH}$. Average the a_{H^+} values, then if you wish, and reconvert to the pH scale. James and others (71) have shown that during the night the human stomach usually has a low acidity (pH 1 to 3) and that during the day the taking of meals is the most frequent cause of changes in acidity of the stomach. As his charts show, however, the high acidity is usually recovered within a short time after the meals are eaten.

In man the duodenal contents are usually in the range pH 5 to 7. This pH range corresponds to from 10 to 0.1 microequivalents of hydrogen ion per L., respectively. There is a gradual decrease in acidity in moving from the duodenum to the ileo-cecal valve, ranging from approximately pH 5 to 6, on the average, in the duodenum to about pH 8 in the lower ileum. This pH range corresponds to a change in hydrogen ion concentration of from about 1 to 10 microequivalents per L. to about 0.01 microequivalents per L. The intestinal pH of the dog is very comparable to that of man (77–79).

From the above it may be concluded that when a drug or its dosage units move from the stomach through the pylorus to the duodenum there is a very abrupt and marked change in acidity in practically all human subjects or patients. The transfer of a given particle, granule, or aliquot of solution containing the drug through the pylorus takes only a fraction of a second. However, as indicated below, the individual particles, granules, or aliquots of the solution from a given dose of the drug may empty from the stomach over a prolonged period of time. The abrupt change in acidity noted above cannot be stressed too much. For example, if the stomach contents have a pH of 1 and the duodenal contents a pH of 6, then the difference in acidity on crossing the pylorus in terms of pH units is 5, but in terms of hydrogen ion concentration is 99,999 microequivalents per L. and the difference in terms of hydroxide ion concentration is 0.009 microequivalents per L.

However, if the duodenal contents have a pH of 6 and the contents of the lower ileum have a pH of 8, then the difference in terms of pH units is 2, but in terms of hydrogen ion concentration is only 0.99 microequivalents per L. and the difference in terms of hydroxide ion concentration is only 0.99 microequivalents per L. Hence, the magnitude of the difference in hydrogen ion concentration between the stomach and the duodenal contents is very large and dwarfs the relatively small change in hydrogen ion or hydroxyl ion concentration between the duodenal contents and the lower small intestine.

Stomach Emptying Rate.—Gastric emptying in terms of emptying time, that is the time taken for the whole or some recognizable constituent of a test meal or dosage form to leave the stomach, does not give much insight into the pattern or kinetics of emptying (80). In 1898 Marbaix, in essence, indicated that the stomach empties by a pseudo first-order process; that is, the volume of meal remaining = (volume at zero time)·$\exp^{-K_e t}$. Subsequently, this has been verified in man (80) and the rat (81). In some cases there is a lag time before the stomach starts to empty; or there may be an initial faster pseudo first-order rate then a slower pseudo first-order rate is subsequently established. James (71) reviewed the following factors influencing emptying of fluid test meals from the stomach of man: (*a*) Serial test meals of different volumes have indicated that once the exponential rate of gastric emptying had been established, the half-lives were longer for larger meals. However, the initial emptying rate was relatively much larger for the larger meals. (*b*)

The exponential rate was not continued to the end for larger meals, but the last portions tended to empty more rapidly. This final phase started when the volume of the meal retained in the stomach was 100 to 300 ml. for the meals of 1,250 ml., but only 20 ml. when the initial volume of the meal was 750 ml. It was not seen at all with meals of 330 ml. initial volume. (*c*) The osmotic pressure of the meal has an effect. For example, addition of sucrose in relatively large amounts to a fluid test meal usually causes a more rapid initial stomach emptying but once the exponential rate is established the rate is much slower when the subjects ate sucrose. (*d*) Cold meals tend to empty faster than hot meals. (*e*) The composition of the test meal influences the rate. (*f*) Most investigators are also agreed that a given individual tends to be true to his type with respect to gastric emptying rate.

Borgström, *et al.* (73), found that a 500-Gm. test meal containing carbohydrate, fat, protein, and water was delivered from the stomach of human subjects to the duodenum in small portions over a four-hour period with a maximum amount during the second hour. Other investigators have obtained similar results (76, 82, 83), although healthy medical students appear to have more rapid stomach emptying rates, probably due to increased mental pressures.

Stomach emptying of larger solid units, such as large granules or tablets, appear to follow the same sort of rules, but often the reported emptying times are longer and over a wider range. If there is only one unit, such as an enteric coated or delayed action (laminated) tablet, we may be dealing with an all-or-none effect. If the single enteric coated tablet stays in the stomach, the patient gets no medicine. If it is laminated, the outer dose dissolves rapidly in the stomach but the inner dose, if protected by an enteric coating, may leave the stomach right away giving a double dose of drug, or it may stay in the stomach anywhere from zero to twelve hours. Hence, the idealistic picture painted for such single unit enteric medication is not supported by the scientific facts. However, if the dose of drug is divided into a large number of units, which stay intact in the stomach, then stomach emptying is gradual (84–86) and extends over a period of time probably comparable to that following a meat meal or longer. The net effect is that medication is delivered to the absorption sites from the time of administration, and continues to be delivered over a considerable period of time postadministration. Blood level studies in man with prednisolone in coated form, where the dose was distributed amongst a large number of individually coated units, indicated the above also (8).

For single enteric coated tablets administered to large numbers of human subjects, and followed by roentgenography or roentgenoscopy, the reviewer has found that most of these sets of data in the literature give one or two linear segments on normal probability or logistic ruling graph paper. In constructing such plots one plots the cumulative percentage of tablets emptied from the stomach on the probability axis, or on the logistic axis, and the time, in hours, on the normal arithmetic axis. The data are usually well represented by only one or two straight lines. If only one line is obtained on logistic ruling paper then the equation of the lines is given by (87):

$$\text{cumulative \% of tablets emptied} = \frac{100}{1 + \text{Antilog}\,(K - t/f)} \qquad \text{(Eq. 25)}$$

where t is the time in hours, K is the time when 50% of the tablets have emptied, and f is the slope factor, or a characteristic of the spread of the plot. The data of Bukey and Brew (88, 89), Crane and Wruble (90), and Blythe, *et al.* (91), obtained by administration of enteric coated tablets to human subjects, and the data of Wagner, *et al.* (92), obtained by administration of such tablets to the dog, all give linear plots on either normal probability or logistic ruling graph paper. These results strongly suggest that the emptying of single enteric coated tablets is just a matter or probability and that one can explain the results by the laws of probability. If one extrapolates this knowledge to the situation discussed above, where a large number of individual units are administered at one time, with the drug dose distributed amongst them, one could deduce that the units would empty from the stomach also according to the laws of probability. If they should empty by a pseudo first-order rate, like liquid meals, this should not be too unexpected since the first-order rate constant is a probability function. In this case, however, medication is being constantly delivered to the absorption sites and one does not get the all-or-none effect observed with enteric coated tablets. Because of the above, it is quite unscientific to average blood levels observed after administration of single enteric coated tablets to large groups of individuals. However, it is perfectly acceptable to average blood levels following administration of a product where the dose is distributed amongst a large number of coated or uncoated units, such as granules. Support for the extrapolation of the data obtained on enteric coated tablets administered to many different subjects to administration of a large number of units which stay intact in the stomach of one individual is given by the data of Deeb and Becker (86). These authors showed that *dl*-amphetamine sulfate coated granules and *d*- and *dl*-amphetamine resin complex administered to rats were emptied from the stomachs of the rats over very long periods of time extending beyond fifteen hours. When their average percentage residual amphetamine in the stomach was plotted on a logarithmic scale against time in hours, the different preparations give two to four linear segments. Even their control, *dl*-amphetamine sulfate administered in 0.5% sodium carboxymethylcellulose, was not emptied completely in an eight-hour period. The authors failed to mention that amphetamine sulfate and sodium carboxymethylcellulose would interact to form a poorly soluble "salt-complex" and that this probably influenced their control results. In fact, one of the sustained action products of amphetamine marketed is just the interaction product cited above. Field, *et al.* (93), working with Resodec, stated that there was a delay in the stomach emptying of the resin in dogs followed by a relatively rapid passage through the intestines. In this case, the ammonium and potassium ions on the resin product Resodec were all exchanged for hydrogen ion while the resinate was in the stomachs of the dogs.

When a sulfonic acid resin and an amine drug are combined to give a resinate the following reactions would be important both *in vitro* and *in vivo*.

$$\underset{\text{amine drug resinate}}{R{-}SO_3^-H_2\overset{+}{N}{-}R'} + X^+ \rightleftharpoons \underset{\text{resin in acid cycle}}{R{-}SO_3^-\overset{+}{X}} + \underset{\text{drug in solution}}{R'{-}\overset{+}{N}H} \quad \text{(Eq. 26)}$$

$$\underset{\text{amine drug resinate}}{R{-}SO_3^-H_2\overset{+}{N}{-}R'} + Y^- \rightleftharpoons \underset{\text{resin in basic cycle}}{R{-}SO_3^-} + \underset{\text{free base of amine drug}}{R'{-}NH_2} + HY \quad \text{(Eq. 27)}$$

Where X^+ is H^+ or another cation; and Y^- is OH^- or another anion.

Similarly when a quaternary ammonium, or tertiary amine type of exchange resin, is combined with an acidic drug to give a resinate, the following reactions would be important both *in vitro* and *in vivo*.

$$\underset{\text{resinate}}{(R)_3{-}\overset{+}{N}H{-}\overset{-}{O}OC{-}R'} + X^+ \rightleftharpoons \underset{\substack{\text{resin in}\\\text{hydrogen cycle}}}{(R)_3{-}\overset{+}{N}H} + \underset{\substack{\text{drug in}\\\text{solution}}}{R'{-}\overset{O}{\overset{\|}{C}}{-}O^-X^+} \quad \text{(Eq. 28)}$$

$$\underset{\text{resinate}}{(R)_3{-}\overset{+}{N}H{-}\overset{-}{O}OC{-}R'} + Y^- \rightleftharpoons \underset{\substack{\text{free base}\\\text{form of resin}}}{(R)_3{-}N} + \underset{\substack{\text{drug in}\\\text{solution}}}{R'{-}\overset{O}{\overset{\|}{C}}{-}O^-} + HY \quad \text{(Eq. 29)}$$

The positions of the equilibria depicted above will depend upon the effective pKa or pKb of the resin, the pKa or pKb of the drug involved, and the concentrations of hydrogen ion and other cations in the stomach and the concentrations of hydroxyl ion and other anions in the intestines. Amphetamine has a pKb of 4.23 at 25° hence will be about 99% ionized in solution at pH 2.23 and about 99% as the free base at pH 6.23. Sulfonic acids are extremely strong acids. One would expect an amphetamine-sulfonic acid resinate to be quite stable in the stomach, which in terms of the above, means that the first equilibrium (Eq. 26) would be expected to be far to the left. However, once the resinate is emptied into the intestine where the pH is between 6 and 8, the second equilibrium above goes far to the right and the amphetamine is released as the free base. Since absorption would occur very rapidly, the titer of free amphetamine base would be very low in the intestinal contents. These considerations possibly aid in explaining the results of Deeb and Becker (86) and of Chapman, Shenoy, and Campbell (25). In the case of creatinine, however, Chapman, *et al.* (25), reported no sustained effect from the resinate. Creatinine is a very weak base with a pKb about 10.5 at 40°. Hence, one would not expect the sulfonic acid-creatinine resinate to be stable at most stomach pH values. Similarly, acetylsalicylic acid has a pKa of 3.49 at 25°. Therefore, even at low pH values observed in the stomach, one would expect much of the acetylsalicylic acid to be displaced from a strongly basic resin by hydroxide ion. Rapid emptying of the acetylsalicylate ion in solution, followed by rapid absorption of the resulting molecules in the intestine, or absorption of nonionized acetylsalicylic acid in the stomach, would reduce the titer of free drug in solution and drive all the drug off the resin quite rapidly.

Other Important Factors.—Mucin may bind some drugs, particularly drugs which are highly basic like quarternary ammonium compounds, and inhibit absorption of the drug (94). Cavallito, *et al.*, made suggestions which may circumvent such binding (95, 96).

The length of the intestinal tract is important in the absorption of drugs since the dose must have time to be absorbed, particularly if the dissolution rate is low. Hirsch, *et al.* (97), reported various intestinal distances which were determined on living human subjects. These are considerably shorter than the classical ones quoted in textbooks which were determined on cadavers. The averages shown in Table II were calculated from these data. Estimates of the surface area involved in the gastrointestinal tract were given by Edwards (65). He estimated that 500 ml. of liquid in the small bowel would occupy a length of intestine of the order of 100 cm. bounded by an area of about 700–800 sq. cm. Since the villi increase the apparent area by a factor of about four, the effective area of absorption in this section is about 3,000 sq. cm. The effective thickness of the epithelial layer may be taken as 25 μ. On the other side of the membrane an adult circulates about 3,000 ml. of blood plasma in about half a minute.

TABLE II.—AVERAGE LENGTHS OF INTESTINAL SEGMENTS OF TEN PATIENTS OF WHOM FIVE WERE OBESE[a]

	Average Values
Body height	163 cm.
Body weight	85 Kg.
Distance from nose to anus	453 cm.
Distance from nose to pylorus	64 cm.
Distance from nose to ligament of Treitz	83 cm.
Distance from nose to ileo-cecal valve	347 cm.
Length of duodenum	21 cm.
Length of jejunoileum	261 cm.
Length of colon	109 cm.
Total small intestine	282 cm.

[a] Calculated from the data of Hirsch, J., Ahrens, E. H., Jr., and Blankenhorn, O. H., *Gastroenterology*, **31**, 274(1956).

The ionic composition of the contents of the various parts of the gastrointestinal tract may be important in the absorption of certain drugs. For example, if the drug formed a nonabsorbable chelate with a metal ion, such as magnesium or calcium, then the amount of drug available for absorption would depend upon concentration of the metal ion, the pH, and the concentration of drug in the tract. The ionic composition of the contents of the various segments of the gastrointestinal tract is shown in Table III (75). Bernstein (74) gives the alterations in electrolyte pattern of the gastric juice in healthy male adults following various stimuli compared with prestimulation levels. The stimuli were insulin, histamine, alcohol-histamine, and adrenalin.

TABLE III.—IONIC COMPOSITION OF BIOLOGICAL FLUIDS

Ion	Blood		Gastric Juice		Bile		Pancreatic Juice		Small Intestine		Large Intestine	
	mg. %	Meq./L.	mg. %	Meq./L.	mg. %	Meq./L.	mg. %	Meq./L.	mg. %	Meq./L.	mg. %	Meq./L.
Potassium	16–22	4.1–5.6	40	10	10–47	2.6–12	11.7–15.6	3–4	15.6–19.5	4–5	33.6	8.6
Sodium	330	143	115	50	326–354	142–154	317	138	322	140	347	151
Calcium	9.0–11.5	4.5–5.8	4.1–8.6	2.0–4.3	4–9	2–4.5	0.4–6.4	2.2–3.2	5.0–12.8	2.5–6.4	10.0	5.0
Magnesium	1.3	0.83–2.5	2.2–9.4	1.8–7.8	1.82	1.5	0.34	0.28	1.2–2.4	1–2	1.95	1.6
Chloride	370	100	500	141	320–355	90–100	213–284	60–80	262–365	74–103	310	87.5
Sulfate	19	4.0	Trace	....	0.3	0.06	39.4	8.2	Trace	....	Trace	....
Phosphate	10	3.1	1.1–4.2	0.34–1.3	12.5	7.3	0.125	0.039	8.3–24.5	2.61–7.66	54.4	17
Bicarbonate	164	27	0–130	0.21	244	40	610	100	12–195	2–32	559	91.8
pH	7.3–7.4		1.2–1.8		7.4–8.5		7.5–8.8		6.16–7.31		8.03	
Titratable acidity or alkalinity	146.6		123.3		151–181		50–160		158.2		164.6	

Copied from "Ion Exchange and Adsorptive Agents in Medicine," by G. J. Martin, Little Brown & Co., Boston, Mass., 1955, pp. 10, 11.

The site of absorption of some drugs is also important. Normal food appears to be absorbed very high up in the small intestine. The absorption of fat, carbohydrates, and protein begins in the distal part the duodenum and is generally complete in the first 50 to 100 cm. of jejunum. The site is a little higher up for fat than for glucose and protein. Thus, the absorption of these foodstuffs occurs in the proximal 20 to 40% of the length of the small intestine (73). Similarly, some vitamins appear to be absorbed in the proximal part of the small intestine. The amount of ascorbic acid absorbed in one hour from a segment terminated 45 cm. beyond the pylorus was not singificantly different than from the amount absorbed in the same time from a segment terminated 90 cm. beyond the pylorus (98). The studies of Campbell, *et al.* (26, 99, 100), indicate that riboflavin is also absorbed in the proximal part of the small intestine and is either decomposed or not absorbed in the distal small intestine or large intestine. Vitamin B_{12}, on the other hand, appears to be absorbed throughout the tract (101) but the fraction of the dose absorbed is very small (102). Bothwell, *et al.* (103), claim that ingested iron is absorbed in the ferrous form largely by the duodenum and that the excretion of absorbed iron into the gut is negligible. Using balance studies on large numbers of children, Schulz and Smith (104, 105) showed that only an average of 10% of iron in food and food supplements was absorbed in normal children; however, iron-deficient children absorbed two to three times as much food iron as did normal children. Similarly, only 12 to 15% of the 30 mg. largest tolerated dose of radioiron ferrous sulfate, given once or twice daily, was absorbed by normal children; iron-deficient children absorbed somewhat more (105). These studies seem to throw considerable doubt as to whether multivitamin products, with and without iron, should be administered in slowly disintegrating or releasing dosage forms, especially of the sustained action type (26). Many drugs, however, are absorbed both in the stomach and throughout the small intestine. This seems to be particularly true of drugs which are weak organic acids, weak organic bases, and their salts (23, 94, 106, 107). Salicylic acid has been shown to be absorbed throughout the tract including the stomach, small intestine, and large intestine (94, 106). The results of Campbell, Nelson, and Chapman (23) indicate that amphetamine, a weak base, is absorbed throughout the small and large intestines; in this case the stomach was excluded by the reviewer because of the results reported by Schanker (94). Nelson (107) has shown that 95 to 100% of an administered dose of tolbutamide is absorbed in human subjects. Similarly, Swintosky, *et al.* (108), reported that nearly 100% of an intravenously administered dose of sulfaethylthiadiazole could be accounted for in the urine within seventy-two hours; when the drug was given as a powder in hard gelatin capsules, an average of about 93% of the oral doses was absorbed. Many neutral molecules, such as the steroids, appear to be well absorbed also.

There have been reports that the rate of absorption of certain compounds is different in different segments of the intestinal tract. For example, Cummins and Jussila (109) reported that glucose and urea were absorbed very much faster in the upper than in the lower small human intestine.

These studies were done by an intubation technique with solutions of the compounds. It is possible that all the authors were measuring were the differences in apparent surface areas of the different segments. No kinetic measurements were done; only the amount absorbed in thirty minutes was determined. Unless kinetic studies are done and the effective surface areas known, one should probably not state that "rates of absorption" are different. It is possible that with solutions of chemicals or drugs, surface area of the absorbing segment is the rate-limiting factor. With fine powders, both dissolution rate and surface areas of the powder and the segment may be involved, but the former is probably rate-limiting in most cases. With restricted surface area of the drug, such as in sustained or prolonged action products, then the surface area of drug exposed and/or rate of diffusion of drug from the product is rate-limiting the absorption process in most cases. If a coating is involved the situation is more complex and much more difficult to interpret.

The actual mechanism of absorption by the intestinal epithelium has been the subject of a great deal of study, particularly in the past few years. The review of Schanker (94) discusses many of these. Apparently, we can exclude active transport when considering the absorption of most drugs. Recently it has been shown that most drugs are absorbed by a simple diffusion mechanism. The absorption of some very large molecules and particles, however, may not occur by a process of simple diffusion. Clark (110) demonstrated that proteins and colloidal materials administered orally to suckling rats and mice were ingested by columnar absorptive cells of the jejunum and ileum, but not by the duodenum. Clark (110) postulated that ingestion of the foreign materials was accomplished by pinacytosis; that is, by invagination of the apical cell membrane to form vacuoles containing material from the intestinal tract. Farquhar and Palade (111) showed that ferritin molecules are absorbed in a similar manner and are transformed into dense bodies or droplets in the size range 0.5 to 2 μ. There have been two reports (112) that insulin, administered orally to suckling rats two to eight days old, causes a large drop in blood sugar levels. The same effect does not occur in twenty-one to thirty day old rats. Both Clark (110) and Farquhar and Palade (111) reported that absorption of the large molecules with which they worked only occurred in young animals also.

The pH-Partition Hypothesis.—In 1902 Overton (113) demonstrated that a number of organic compounds penetrate cells at rates roughly related to their lipid/water partition coefficient. He concluded that the cell membrane is lipoidal in nature. For the next fifty-five years the literature concerning absorption and transport appeared to be mainly concerned with active transport and facilitated diffusion. In 1937 Hüber and Hüber (114) reported that certain groups of similar compounds were absorbed from intestinal loops of the rat at rates roughly proportional to the size of the molecules. However, in each of these groups the rates of absorption were also related to the relative lipid/water partition coefficients. The absolute amount of polyhydric alcohol or aliphatic acid amide absorbed was directly proportional to the concentration in the intestinal lumen; the percentage absorption remained constant over a wide range of concentrations. These and other experimental data indicated that the foreign molecules were absorbed from the intestine by a process of simple diffusion through a lipoid-sieve type of boundary. Travell (115) in 1940 noted that large doses of alkaloids produced no toxic effects when the gastric contents were highly acidic. When the gastric contents were made alkaline the animals rapidly died, indicating that only the undissociated free bases of the alkaloids were absorbed. This was the first indication that the gastric epithelium is selectively permeable to the undissociated form of a drug. Later, Shore, *et al.* (116), showed that parenterally administered drugs are secreted directly into gastric juice. The concentration ratio (concentration of drug in gastric juice divided by concentration of drug in plasma) depends on the dissociation constant of the drug. Strong organic acids appear in gastric juice in negligible concentrations, weak acids and bases in measurable concentrations, and strong bases in highest concentration. The amount of the strongest organic bases transferred from the plasma to the gastric juice is limited by the rate at which the drug is delivered to the gastric mucosa, i.e., by the rate of gastric mucosal blood flow. The data of Shore, *et al.* (116), were explained by the concept that the membrane separating plasma from gastric juice has the characteristics of a lipoid membrane that allows the passage of drugs in their undissociated form while restricting passage of the dissociated form.

Brodie, Hogben, Schanker, Shore, and Tocco (117–121) developed the pH-partition hypothesis which is a great advance in explaining the rate and extent of absorption of acidic and basic drugs from the stomach and intestines of the rat and the human being. Their investigations and results were recently reviewed by Schanker (94) and will not be extensively discussed in this review. The concept has also been applied to the kinetics of penetration of drugs and other foreign compounds into cerebrospinal fluid and the brain (122).

Prior to the perfusion experiments in rats carried out by Brodie, *et al.* (117–121), the intestine was cleared of all particulate matter by perfusion with drug-free solutions for thirty minutes, and then perfused with drug solution for thirty minutes to displace the washings. Although it was not mentioned by the authors, such treatment would most probably remove some of the mucus or mucosubstance (123) from the stomach wall and intestines. Their results would not include the effects of interaction of a given drug with mucus. The effect of food on absorption of a given drug is also excluded by the nature of their experiments.

Complete understanding of the pH-partition hypothesis requires reading the original manuscripts of Brodie, *et al.* (117–121). Only a few of the most important aspects will be discussed here. For most drugs with molecular weights greater than 100, penetration through pores is relatively unimportant (119). Foreign organic substances penetrate the mucosa as though the boundary had the characteristics of a lipoid barrier. The passage of drugs across these barriers is governed mainly by physical processes and is predictable from the dissociation constants and the lipid-solubility of the undissociated moiety (120). The drugs are absorbed by the passive diffusion of the unionized form. In certain cases, such as quaternary ammonium compounds,

the intestinal mucosa may be slightly permeable to organic ions (120). The pH at the site of absorption is not necessarily the same as the pH of the contents in the center of the intestinal lumen. Hogben, *et al.* (120), calculated a "virtual" pH at the site of absorption of 5.3, although the pH of the perfusion fluid on leaving the intestine was 6.6. This "virtual" pH is to a considerable extent, but not completely, independent of the pH of the bulk solution within the lumen. In the case of solutions of drugs in the intestines, diffusion from the center of the intestinal stream to the intestinal wall becomes the rate-limiting step when absorption is very rapid. For this reason the proportion of unionized moiety only becomes a rate-limiting factor when it is less than a critical value. For example, in the case of salicylic acid the permeability of the mucosa is such that the critical proportion is unionized : ionized = 1 : 200. When the ratio is greater than 1 : 200, the maximal rate of absorption of salicylic acid is attained (120). The relationship between the lipid solubilities of the unionized drug moieties and their rates of intestinal absorption suggests that the intestinal blood barrier is lipoidal in nature. This is clear-cut for the highly lipid-soluble and very poorly lipid-soluble drugs but is not as clear-cut for compounds of intermediate lipid-solubility. The reason for the latter may be that the solvents used to estimate the partition coefficients (chloroform and heptane, for example) are not the proper solvents. We need to know more about the boundary since any one solvent would not be expected to be like the boundary (120).

Schanker, *et al.* (119), pointed out that further evidence of a physical process in absorption was seen in the failure of one drug to alter the absorption of another. They indicated that drug binding to nondialyzable materials in the intestinal perfusate was less than 4% for most of the drugs investigated. However, true complex formation between two drugs, or a drug and an adjuvant, may be expected to alter absorption rate since the diffusion coefficients and solubilities of the complexes may be different than the drug itself. Thus, individual drugs should be studied as individual entities. One cannot generalize.

The pH-partition hypothesis was applied to the absorption of heparin recently (124, 125). When the initial pH of the intestinal lumen of the dog was about 4.0, absorption of approximately 10% of instilled heparin occurred. During the experiment the pH returned to near neutrality and absorption ceased. The authors claimed that under normal circumstances heparin absorption would be expected to be limited to the stomach. Salafsky and Loomis (125) partially esterified heparin with methanol to give a product still possessing anticoagulant activity. Perfusion studies on this compound showed limited but appreciable absorption of the ester did occur from the duodenum.

Although amine drugs are transferred readily from plasma to gastric juice, as indicated above, the actual amounts present in the stomach at any given time is small compared to the total amount in the body since the volume of fluid in the stomach is very small compared with the volume of the other fluids of distribution. The quantitative aspects of the diffusion of weak acids and bases from plasma to gastric juice and urine was reviewed by Milne, Scribner, and Crawford (126). Further application of the pH-partition hypothesis is presented in the section on Percutaneous Absorption.

Factors Affecting Rate and Extent of Absorption Under Normal Physiologic Conditions in Man and Animals.—A review of the literature (127) indicated that the following factors may possibly influence rate and extent of intestinal absorption. Factors involving the membrane or barrier: (*a*) special properties or "vital functions" of the epithelial cell; (*b*) permeability of the membrane; (*c*) electrical phenomena (the magnitude and sign of the charge); (*d*) segmental activity of the bowel; (*e*) the absorbing surface area per unit length of gut; (*f*) the degree of villous activity; (*g*) the degree of vascularity. Factors involving the substance absorbed: (*h*) the geometry and functional groups of the substance; (*i*) the presence or absence of a charge and its sign and magnitude; (*j*) the diffusibility of the solute; (*k*) the solute concentration on the two sides of the membrane; (*l*) the molecular size or molal volume of the solute; (*m*) the particle size of the substance and its physical state; (*n*) the lypophilic and hydrophilic properties of the substance; (*o*) the solubility and rate of dissolution of the substance. Miscellaneous factors: (*p*) the temperature; (*q*) the gradient of absorbability down the tract; (*r*) hydrostatic and intraintestinal pressure; (*s*) surface and interfacial tension; (*t*) pH of the intestinal contents; (*u*) enzymatic activity; (*v*) the concentration of simple ions in the intestinal contents; (*w*) the presence or absence of mucus, complexing agents, emulsifying agents, etc.; (*x*) the gross anatomical position and relative activity of the subject; (*y*) competition between molecules for absorption; (*z*) the emptying rate of the substance from the stomach.

Many of these factors may be operative during the absorption of a given drug under normal conditions of its use in man. To study properly the relative importance of some of these one must control certain variables during experiments and investigate the effects of certain changes in other variables, such as was done by Brodie, *et al.* (107–121), in developing their pH-partition hypothesis for acidic and basic substances. Many clinical and pharmacologic reports in which the absorption of two or more drugs, or the absorption of one drug in the presence or absence of an adjuvant, have been compared are deficient in specifying important facts which may have a bearing on the interpretation of the results. In many cases authors have reached conclusions which are not justified by the data they present. A paper presented by Nelson (128) at a recent symposium, which will be published in the near future, reviews a number of such reports. Hence these will not be considered in this review. Instead, some examples where a given variable has been isolated and shown to be important in the absorption of the substance will be presented.

Hydrolysis of Substances in the Gastrointestinal Tract.—If the drug contains a linkage susceptible to hydrolysis it may, or may not, be hyrolyzed before absorption. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were studied by Okuda (129). Homogenates of the small intestine rapidly dephosphorylated FMN probably due to phosphomonaesterase in the mucous mem-

brane. FAD was decomposed also to FMN and riboflavin. In pancreatic juice, free riboflavin was not decomposed but FMN was about 45% dephosphorylated to free riboflavin during two hours incubation at 37°. Long, *et al.* (130), reported that the limiting factor governing absorption of propylene glycol distearate in the rat is the hydrolysis rate of the ester in the tract. Similarly, Vahouny and Treadwell (131) reported data which was in agreement with the concept that dietary cholesteryl esters are hydrolyzed in the gastrointestinal tract prior to absorption of the cholesterol moiety as free cholesterol. Glazko, *et al.* (132), did an excellent study with various esters of the antibiotic chloramphenicol. They found that the esters had to be hydrolyzed enzymatically before absorption occurred; their data indicated that dissolution rate and surface area were rate-limiting the absorption process. It was reported that the best blood levels were produced by dissolving chloramphenicol palmitate in dimethylacetamide and injecting the solutions forcibly into equal volumes of water containing 10% of polysorbate 80. Under these conditions one would expect an extremely fine dispersion of the ester with a large surface area. On the other hand, Blough, *et al.* (133), reported that in man 80% of the propionyl erythromycin excreted in the urine exists as the intact but inactive ester. Cope and Black (134) stated that C^{14}-cortisone acetate is absorbed and excreted at almost the same rate as C^{14}-cortisol; and that once absorbed the former is readily converted into the latter. The paradoxical dependency of the effectiveness of Δ^4-3-keto steroidal enol ethers on the route of administration was reported by Ercoli and Gardi (135). The ethers were stated to have enhanced oral activity compared with the parent hormones, but greatly decreased parenteral activity. These authors failed to mention the possibility that there may be a difference in hydrolytic rates of these ethers in the gastrointestinal tract compared with that in parenteral depots and that this may possibly explain the differences.

Dissolution Rate.—In a recent review (136) it was stated that: "different salts of the same drug rarely differ pharmacologically; the differences are usually based on physical properties." The authors then cited examples, and, unfortunately, included chloramphenicol palmitate, which is an ester and not a salt. Perhaps qualitatively, i. e., in the nature of the biological response elicited, a series of salts of the same acidic or basic drug may not differ appreciably. However, quantitatively there may be vast differences. The observed difference between a weak acid or base and its salts, or between different salts, will be the intensity of biological response in relation to time after administration. This must follow from the fundamental factors considered before. Each drug must be studied as a separate entity and the effects of administration of the weakly acidic or basic drug itself, and of various salts, observed. Few generalizations can be made because of the large number of variables involved. However, as Nelson (64) pointed out, the dissolution rate of the particular form largely determines the rate of build-up of blood level with time and the maximum blood level attained. Nelson (128) reported that, *in vitro*, the initial dissolution rate of tolbutamide sodium in acidic medium was nearly ten thousand times more rapid than tolbutamide in the same medium. In buffered mediums of near neutral pH about a two hundredfold difference existed in favor of the salt. These large differences measured *in vitro* reflect the differences observed between tolbutamide sodium and tolbutamide *in vivo* when administered to normal human subjects as shown in Fig. 3 (137). Tolbutamide sodium, administered orally, produces a very rapid fall in blood sugar comparable to that produced when the same salt is administered intravenously to normal subjects (138). The more slowly dissolving weak acid, tolbutamide, administered as the commercial compressed tablet, produces a smooth, sustained fall in blood sugar level, as the plots show. From 95 to 100% of the doses of both tolbutamide, administered as the commercial compressed tablet, and its sodium salt, administered as compressed tablets, are absorbed following oral administration (107). Similar effects of different salts of theophylline (64), tetracycline (66), and penicillin (63) were discussed before. Lee, *et al.* (139), reported that the potassium salt of penicillin V produced higher and earlier blood levels and was better absorbed in the stomach than the free acid. Wright and Welch (140) reported similar results in fasting human subjects. This undoubtedly reflects large differences in dissolution rates of the potassium salt and the free acid in the stomach contents.

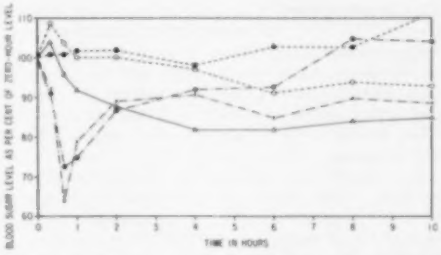


Fig. 3.—Illustration of the effect of difference in dissolution rates of a weak acid and its sodium salt on the nature of the biological response-time plot. Day No. 1, O- - -placebo, six normal subjects; △—— C. T. Orinase, 1.0-Gm. dose administered as two tablets, 10 normal subjects; x ····· C. T. Orinase sodium, dose equivalent to 1.0 Gm. of Orinase administered as four tablets, 5 normal subjects. Day No. 2, ——— placebo, five normal subjects; x····· C. T. Orinase sodium, dose equivalent to 1.0 Gm. of Orinase administered as four tablets, seven normal subjects.

In some cases physical form is exceedingly important making it difficult, as stated above, to write generalizations. An example is the report of Mullins and Macek (141) concerning various forms of novobiocin. These authors reported that crystalline novobiocin acid, at a dose of 12.5 mg./Kg. in dogs, produced no detectable plasma levels of the antibiotic over a six-hour period. Their *in vitro* data indicate that in 0.1 *N* hydrochloric acid this crystalline acid dissolves exceedingly slowly. However, amorphous acid novobiocin, administered orally in the same dose, produced peak blood levels and an area under the blood level-time plot, approximately twice that produced by the same dose of the sodium salt. Hence, apparently, amorphous

acid novobiocin is both much more rapidly and much more efficiently absorbed than the sodium salt in dogs. However, the amorphous acid slowly converts to the crystalline acid in aqueous solutions making readimixed suspensions of the amorphous acid impractical for commercial sale. Fúrész (142) obviously tested crystalline novobiocin since he reported negligible levels in human subjects; he failed to distinguish whether he was testing the crystalline or amorphous form.

Differences in dissolution rate *in vivo* of different brands of dosage forms of the same drug have considerable therapeutic implication. This aspect of the problem was discussed by Levy recently (143). Everyone who fosters indiscriminate brand interchange should read his article and check the references. The reviewer was rather amazed to see the conclusions drawn in a recent review (144) concerning prednisone tablets. A partial excerpt is as follows: "While the disintegration test (U. S. P. test) is a severe one, the failure of the first samples . . . to disintegrate under the test conditions might in some instances be reflected in reduced absorption of the drug. In general, however, the risk involved in prescribing prednisone by generic name appears to be small." Drawing such conclusions without biological data can be exceedingly dangerous and misleading. Even the statement that the U. S. P. disintegration test for compressed tablets is "a severe one" needs comment. In a group of commercial acetylsalicylic acid tablets tested by Levy and Hayes (145, 146), the most rapidly dissolving products exhibited longer disintegration times than the slowly dissolving products. The authors concluded that disintegration times is no index of rate of dissolution or of availability of the medicament. Their *in vitro* work was supported by *in vivo* tests in which quantitative measurements of excretion of salicylate were made in a large number of human subjects after oral administration of different commercial aspirin tablets. They found that the absorption of aspirin is affected by: (*a*) the dissolution rate (not the disintegration time) of the tablets, (*b*) the gastric emptying rate, and (*c*) the mode of administration. They even found that doubling the amount of water taken with the tablets vastly altered the rate of absorption of the aspirin. Their recommendation, based on the overall results, was that the U. S. P. disintegration test be replaced by a dissolution test. A disintegration test merely measures the time required for the tablet to break up into a certain size range of granules which will drop through the certain size of screen in the apparatus. The test tells nothing about how rapidly the drug will be released from the small granules. Many substances, such as cement or metals, could be made into granules and compressed into tablets which would pass the U. S. P. disintegration test; *in vivo* the granules would pass through the intestinal tract completely unchanged and be excreted in the feces.

A problem of introducing a dissolution test to replace the disintegration test for tablets is the additional time needed for control laboratories to run the former compared with the latter test. A possible means of circumventing this is to correlate the results of a dissolution test with a disintegration test on each product. An example is shown in Fig. 4. In the example cited it is shown that in four out of five tablets tested, the disintegration time corresponded to between 90 and 100% of the drug being in solution; for the fifth tablet, tablet D, the disintegration time corresponded to about 80% of the drug being in solution. With such a correlation it may be valid to use the disintegration test as a routine procedure. However, such a correlation would have to be established for each drug formulated in tablets and made by a certain procedure. One should not extrapolate the results shown in Fig. 4 to a totally different drug or to tablets made by a different manufacturer.

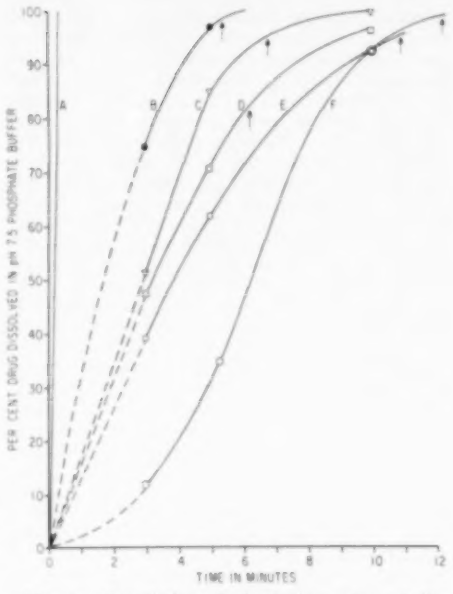


Fig. 4.—Correlation of dissolution rate and disintegration time. Curve *A* is the powdered drug. Curves *B*, *D*, and *E* are different lots of uncoated tablets of the same drug. Curves *C* and *F* are two lots of coated tablets of the same drug. The arrow on each dissolution plot corresponds to the disintegration time of the tablets as determined by the U. S. P. procedure.

A good pharmaceutical house tests its product *in vivo* and establishes that the particular dosage form does give the response expected. One cannot predetermine such an effect from *in vitro* experiments but only make "guessestimates." Because of these considerations the reviewer and others (147) believe it is equally dangerous to assume that a sustained action preparation will not work *in vivo* based upon results of some *in vitro* tests, such as is apparently done sometimes by the Food and Drug Administration (148). Unless a correlation has been established for the given drug in the given dosage form between the *in vitro* test results and the *in vivo* results, one is only guessing.

Alteration in the surface area of a dose of a medicament can have an effect similar to changes in type of salt or physical form. This follows from a consideration of the equations describing dissolution rate as a function of several variables above. Mouriquand, *et al.* (149), gave evidence that there

was a difference between ordinary and lyophilized thiamine hydrochloride when tested in pigeons. A given weight of lyophilized thiamine hydrochloride would be expected to have a very much larger surface area than the same weight of crystalline thiamine hydrochloride. In fact, such differences in surface area account for the very rapid *in vitro* dissolution times of many commercial parenteral products which contain lyophilized powders. The report of Sakuma, *et al.* (150), stated that absorption of sulfadimethexine was more rapid when the drug was administered in microcrystalline suspension than when the same dose was administered as compressed tablets. Their blood level data also indicated that a greater percentage of the dose was absorbed following administration of the microcrystalline suspension than following the tablets since there was a considerable difference in the areas under the average plasma level-time plots. Hence, it would appear that this drug's absorption is rate-limited by surface area. Reporting on his tests of a sustained action preparation, King (151) wrote: "A markedly greater therapeutic effect was obtained when the tablets were chewed before swallowing. This confirms the experience of Weiss and his co-workers and is now a routine recommendation in prescribing this preparation." In the same article it was stated that: "according to the manufacturers the—[drugs]—are incorporated in an inert binding material from which they are released slowly in order to extend the duration of their therapeutic activity." It appears that in this case the clinician did not think when he wrote his article. Chewing such a sustained action product before ingesting it would almost, or completely, destroy the sustained release mechanism; and, if the dosage form contained two or three usual doses of drug, obviously allow rapid absorption of the total drug.

Effect of Adjuvants on the Absorption of Drugs.—In the recent literature there have been many reports that the administration of a certain compound, hereafter called the adjuvant, will increase the fraction of the dose of a certain drug which is absorbed, or at least produce higher peak blood levels of the drug. In many cases the adjuvant is the solvent, or a component of the vehicle, in which the drug is administered. Many or all of these may be real effects. However, usually insufficient control of variables, inadequate description of the experiments, and/or the forms studied throw considerable doubt on the conclusions drawn. Furthermore, the effect of an adjuvant may be on the blood side of the gastrointestinal barrier, after absorption of both drug and adjuvant into the blood stream occurs, and and have little or nothing to do with absorption, *per se*. Often such possibilities are not considered by the authors. The reviewer has collected about two hundred such references, but no attempt to review all of these will be made. Some of the complicating factors in such studies will be discussed however.

Snell and English (152) reviewed some of the possible reasons for the increased serum levels of tetracycline hydrochloride following oral administration of the antibiotic with glucosamine. The possible reasons cited were: (*a*) increased absorption from the gastrointestinal tract; (*b*) decreased elimination in the bile, urine, and feces; (*c*) alteration in binding equilibria between cells, protein, and aqueous phases of blood; (*d*) alteration in cell permeability in favor of extracellular spaces; and (*e*) alteration of liver metabolism whereby less of the absorbed antibiotic is metabolized by this organ. Bunn and Cronk (153) reviewed tetracycline blood levels obtained in five laboratories and concluded that "tetracycline phosphate complex, tetracycline base with sodium hexametaphosphate, tetracycline hydrochloride with citric acid, and tetracycline hydrochloride with glucosamine hydrochloride produced significantly higher serum concentrations than those obtained with tetracycline hydrochloride within the early hours after administration." However, Finland (154) in an editorial in the same issue stated: "The paper of Bunn and Cronk in this issue unfortunately gives the erroneous impression that by lumping together large amounts of heterogeneous data one arrives at a true evaluation of differences. Far from being true, this is a well-known and obvious technique for submerging true differences or similarities through dilution of well-controlled data with large numbers of observations that are not so well controlled." The carefully controlled experiments of Nelson (155) indicated there were no significant differences in absorption of tetracycline hydrochloride when administered as a 200-mg. dose alone, or with various potentiators, including hexametaphosphate, citric acid, and glucosamine hydrochloride, as judged by urinary excretion of unchanged tetracycline. It would appear that this controversy is still far from solved.

Okuda, *et al.* (156), presented data which they claimed clearly demonstrated that supplementation of a vitamin B_6 deficient diet with D-sorbitol brings about an increase in the urinary excretion and in the liver concentration of vitamin B_6 in young adult rats. They stated that the results may be interpreted as evidence that the absorption of vitamin B_6 was increased by the sorbitol. The effects of D-sorbitol on vitamin B_{12} absorption, under similar conditions, were different than those with vitamin B_6. Prolonged feeding of D-sorbitol resulted in apparent reduction of vitamin B_{12} absorption with concomitant decreases in the plasma and liver concentrations of vitamin B_{12}. Their results seem to contradict the previously reported observations on increased absorption when vitamin B_{12} was coadministered with D-sorbitol by mouth to normal rats and human volunteers. However, they claimed these diametrically opposite results may be explained on the basis of differences in the physical states of the two mixtures of vitamin B_{12} and sorbitol and in the manner in which the experiments were performed. Their unpublished data indicate that sorbitol enhanced absorption of vitamin B_{12} only when a large dose (1,000 mcg.) of vitamin B_{12} was used, and that without the cecum this effect cannot be shown in rats. The markedly enlarged ceca of the sorbitol-dosed rats suggest a possible role of the cecum in the absorption of vitamin B_{12}. They also pointed out that in their study the absorption of vitamin B_{12} was measured under a condition in which sorbitol was not present in the lumen when the test dose was administered. Such a condition permits estimation of the effect of prolonged feeding of the adjuvant on the absorption capacity of the intestinal wall, rather than a direct effect of sorbitol

on absorption of vitamin B_{12} or interaction between the two components.

Nontoxic doses of the surfactant, sodium lauryl sulfate, greatly increase the rate of absorption of glucose administered to rabbits according to Kozlik and Mosinger (157). Subsequently, Hardt (158) claimed that sodium lauryl sulfate introduced in certain doses in solid or solution form into the stomachs of dogs produces complete inhibition of normal gastric motility for periods up to ninety minutes. Later, Nissim (159) reported that both trimethylhexadecylammonium bromide and stearate inhibited the absorption of glucose but did not inhibit the absorption of methionine or sodium butyrate in the rabbit. He claimed his results supported earlier conclusions that large doses of ionic surfactants lead to structural damage, while small doses appear to reduce the functional efficiency of the mucosal cell. Careful reading of these papers indicates that further work is necessary to elucidate the effects of ionic surfactants on absorption.

Sometimes artificial conditions introduced during experimentation cause a different effect than that observed under normal conditions. For example, Raven, *et al.* (160), found that lysine markedly increased the deposition of single doses of Ca^{45} and Sr^{85} in the femur of fasted rats, but supplementation of a normal diet with additional lysine did not bring about a similar effect. Furthermore, they found that glutamic and aspartic acids markedly reduced the enhancement of absorption of Sr^{85} and Ca^{45} produced by lysine. Chelation between glutamic acid and calcium was postulated to account for the suppression. The presence of casein and starch also largely suppressed the effect of the lysine on absorption of the metallic ions. Similarly, the presence or absence of food in the gastrointestinal tract has a pronounced effect on the observed blood levels obtained after administration of certain antibiotics (161). Most clinical experiments, where blood levels of antibiotics are measured, are carried out with fasting patients or subjects.

The effect of an adjuvant on the bacterial flora of the gastrointestinal tract may also be responsible for alteration in the absorption of a drug or food ingredient. Samuel (162) reported that large doses of neomycin, administered orally to human subjects, produced a 17 to 29% decrease in mean serum cholesterol levels. Intramuscular injection of neomycin failed to have the same effect. He reasoned that since only about 3% of an oral dose of neomycin is absorbed, the neomycin must act in the tract. It was suggested that the effect may be due to modification of the flora or inhibition of intestinal enzymes. Since cholesterol esters must be hydrolyzed, apparently, before they are absorbed, this could be the explanation. However, one would have to exclude the possibility that neomycin and and either cholesterol esters, or cholesterol, formed a less soluble complex.

The effect of buffering agents on the absorption of weak acids, such as acetylsalicylic acid and alkaloidal salts, has been the subject of extensive investigation. Rapp, *et al.* (163), found that repetitive administration of 2 mg. of lobeline sulfate, administered in combination with small amounts tricalcium phosphate and magnesium carbonate, produced average lobeline plasma levels which built up from 0.3 to 1.8 mcg./ml. over a six-day period. The same dose of the alkaloidal salt administered without the buffer salts gave essentially no plasma levels, and the placebo gave none. Such effects are difficult to understand in the light of the report of Rubin, *et al.* (164). The latter authors found that amounts of antacids administered with aspirin preparations had insufficient buffering capacity to cause a significant reduction in gastric acidity in human subjects. One would not expect, on the basis of their report, that the amount of antacid administered with the lobeline sulfate would significantly alter the pH of the gastric contents either. Perhaps further investigation in this area is necessary.

When Case, *et al.* (165), described a new technique for preparing sterile pellets for implantation in experimental animals they did not mention the possible effects of their adjuvants, namely 45% lactose and 5% acacia, on the dissolution rate *in vivo*. One would expect a considerable difference in dissolution rate *in vivo* from a pellet prepared as they recommended and a pellet of pure compressed methyl cholanthrene. The effect of the adjuvants may be different for different carcinogenic agents also. The presence of the adjuvants in this case would alter the effective surface area of the carcinogenic agent in the depot. There may also be an interaction effect. For example, Wells, *et al.* (166), reported that lactose had a stimulating effect upon cholesterol absorption. This is another example where biological testing, and comparison of results from the new formulation and a control, should have been made.

In some cases an adjuvant may inhibit the absorption of a compound by formation of a less soluble compound. Hudson, *et al.* (167), reported that sitosterol and cholesterol formed a 1:1 mixed crystal or "solid solution," which has a solubility only one-third that of cholesterol in methanol and a reduced solubility, compared with cholesterol, in aqueous sodium oleate or sodium desoxycholate solutions. This may possibly explain the hypocholesteremic effect of sitosterol in the human being.

RECTAL ABSORPTION

Until 1958 pharmaceutical research and development directed towards rectal administration of drugs was principally concerned with modifying the properties and physical characteristics of suppository bases. *In vivo* studies following rectal administration of drugs were usually of the type where a pharmacological response, or blood, or urine levels, were measured at one or more times. Such studies are based on the assumption that the rate of rectal absorption bears a constant, direct relationship to the amount of drug in a certain body fluid or organ, or the blood level required to elicit a pharmacologic response.

In 1958 Riegelman and Crowell (168) published the details of a radiological procedure by which it is possible to conduct continuous external detection of the rate of rectal absorption of radio-tagged compounds from specially designed suppository vehicles inserted in the rectal passage of the female rat. The experimental design is such that a diffusion gradient is set up within the rectal section. The

section simulated a finite cylinder with sealed end faces, diffusion out of the cylinder occurring in a radial direction only. Using the mathematical solution of Dünwald and Wagner (169) for radial diffusion, the authors were able to derive a relatively simple equation to evaluate their data. This equation is:

$$(\log N) - (N_f/N_0) - N_f = -kt \text{ (Eq. 30)}$$

where N = the dose detected by the external counter at time t, N_f = the dose detected by the counter at the end of the experiment, N_0 = the total dose administered as detected by the scintillation counter, k = a pseudo first-order rate constant with dimensions, time^{-1}, t = the time.

The derivation includes no mathematical considerations of the rate process for drug transfer across the rectal membrane. In general, they believed that the absorption rate process was very much faster than the diffusion process. The apparent rate constant, k, is complex in nature, including diffusion, absorption, and differences in formulation. However, it is extremely useful in expressing the data and making accurate comparisons of variations in formulations.

Applying their method to various compounds and suppository vehicles, Riegelman and Crowell (168) concluded the following: (*a*) The rate of absorption of sodium iodide is accelerated in the presence of surfactants and appeared to be proportional to the surface tension lowering and the peptizing action of their surface active components. (*b*) The rate of absorption of the sodium salt of 2,4,6-triiodophenol is retarded by the presence of surfactants. Hydrolysis and subsequent solubilization of the free phenol was postulated to explain this effect. (*c*) I^{131}-tagged iodoform and 2,4,6-triiodophenol were best absorbed from true solutions in water, or from aqueous suspensions. Surface active agents and polyoxyethylene polymers markedly retarded the rate of absorption of these agents. The relative particle size of these drugs was also shown to affect the rate of absorption. Solutions of the compounds in solid polyethylene glycol and oleaginous bases resulted in very prolonged absorption times.

Samelius and Astrom (170) reported that administration of 0.75 Gm. of acetylsalicylic acid in two types of suppository bases by the rectal route to human subjects resulted in essentially equivalent blood levels of salicylate to those obtained with the same dose by the oral route of administration. They also observed that rectal absorption of hexobarbital sodium was better from cocoa butter base than from a Carbowax base in the rabbit.

Reporting on a study done in rats in which the colon was ligated near the anus and near the ileocecal junction to form sacs, Lish and Weikel (171) found that: (*a*) absorption of phenol red (an acidic indicator) was enhanced by the presence of anionic surfactants and that this enhanced absorption was retarded by treating the rats with chlorisondamine or atropine; (*b*) the absorption of phenol red was not affected by the nonionic surfactant, Pluronic F-68; (*c*) the absorption of methyl violet (a basic indicator) was not affected by the anionic surfactants, Aerosol OT or sodium lauryl sulfate.

PERCUTANEOUS ABSORPTION

The primary requirement for topical therapy is that the drug incorporated into a vehicle must reach the skin surface at an adequate rate (172) and in sufficient amounts. Although dermatologic vehicles may not penetrate the skin to any extent nor act as carriers to transport the medicament through the epidermal barrier, there may be a marked difference in the clinical effectiveness of a drug when using different vehicles (172). The choice of vehicle will depend upon both the characteristics of the drug and the nature of the condition to be treated. This section will be concerned with some of the fundamental factors involved in the rate and extent of percutaneous absorption. Then we will discuss some other factors which are capable of altering the fundamental factors.

Fundamental factors involved in rate and extent of percutaneous absorption are: (*a*) type of skin and whether the skin is normal, abraded, or diseased (173); (*b*) site of absorption—transfollicular route, transepidermal route, or both (173, 174); (*c*) effective thickness of the skin barrier phase (174); (*d*) area to which the drug is applied (173, 174); (*e*) thermodynamic activity of water in the vehicle and in the skin barrier phase (174); (*g*) thermodynamic activity of the drug in the vehicle (174); (*h*) thermodynamic activity of the drug in the skin barrier phase (174); (*i*) diffusion coefficient of the drug in the vehicle (174); (*j*) diffusion coefficient of the drug in the skin barrier phase (174); (*k*) individual contact time and the frequency of reapplication (173).

Shelmire (173) pointed out that the vehicle assumes more importance when the stratum corneum is intact and that differences in drug penetration attributable to the vehicle are more pronounced. On abraded or diseased skin there may be a large increase in both rate and extent of absorption of a drug in a vehicle (173). The site of absorption may be important since a vehicle such as petrolatum may plug the follicles and delay penetration of the drug (173). One would expect the rate of absorption to be inversely proportional to the thickness of the skin barrier (173, 174). Similarly, one would expect the extent of absorption to be directly proportional to the area of skin covered by the ointment, cream, or lotion (173, 174).

Shelmire (173) stated that hydration of the stratum corneum is one of the most important factors in drug penetration into the skin. He (173) hypothesized that the "outer" stratum corneum may be considered a semisolid acid which ejects protons absorbed on the surface, and itself becomes negatively charged relative to the surrounding positively charged solution. The hydration of the stratum corneum results from water diffusing from the stratum mucosum, from water diffusing in from the atmosphere, or from perspiration that accumulates after application of an occlusive vehicle on the surface (173). As Higuchi (174) has pointed out, however, the important thing is the thermodynamic activity of water in the barrier phase, not just the amount there. The activity of water may be altered, for example, by neutral salts. Shelmire (173) stated that hydration of the stratum corneum appears to increase the rate of passage of all substances which penetrate the skin. He (173) suggested the mechanism was to increase the size of

the pores. There will not only be a physical alteration of the tissue due to hydration but also at high water activities there will be changes in both the diffusion coefficient and activity coefficients of the penetrating agent (174). The nature of the vehicle upon application and, when the water in it has evaporated, may markedly alter the activity of water in the stratum corneum. Greases and oils are the most occlusive vehicles and induce increased hydration through perspiration accumulation at the skin-vehicle interface (173). Humectants in vehicles tend to decrease the extent of hydration of the stratum corneum by interference with the formation of a continuous oil film on the skin surface (173). Hydrophilic powders will decrease the extent of hydration by increasing surface area and, hence, increasing rate of evaporation of water (173). If an ointment is covered with a bandage there will be a tendency to hold perspiration and increase hydration; if left open to the air the perspiration can evaporate and dehydration may occur (173). Similarly, the thickness of the applied film of ointment or creams will directly affect hydration of the stratum corneum (173). If one assumes that the effective thickness of the hydrous barrier depends upon the rate of flow of blood through it, then capillary dilation and rate of capillary blood flow will influence absorption (174).

One thing that the pharmacist can alter is the thermodynamic activity of the drug in the vehicle. The thermodynamic activity of the drug in the vehicle is the product of the concentration of drug and the activity coefficient of the drug in the vehicle. Many authors have indicated the importance of the concentration of drug in a topical preparation, but few have indicated that the activity coefficient may be changed in many ways. Some investigators have increased the concentration of the drug in the vehicle but actually have lowered thermodynamic activity because they are apparently not aware of the effect of pH and other factors. Higuchi (174) pointed out that the driving force behind the drug movement is the difference in the thermodynamic potential between the vehicle and the deeper tissues and the direction of flow for systems is always from higher thermodynamic potential to lower thermodynamic potential.

Absorption from Suspensions.—Consider the case of an extremely fine dispersion of the drug in a homogeneous base, such as penicillin in a petrolatum base. Higuchi (174) derived the following simple relationship among the variables:

$$Q = \sqrt{Dt(2C - C_s)C_s} \qquad \text{(Eq. 31)}$$

where Q = the amount of drug absorbed at time t per unit area of surface exposure, C = the concentration of the drug in the vehicle, e. g., in mg./cm.^{3}, C_s = the solubility of the drug in the external phase of the vehicle, D = the diffusion constant of the drug molecule in the external phase of the vehicle, and t = the time, e. g., in hours.

The derivation involves the assumptions that release by the ointment itself is rate-limiting the absorption process and that the skin surface constitutes a perfect sink for the released drug.

$$\frac{dQ}{dt} = \frac{1}{2}\sqrt{\frac{D(2C - C_s)C_s}{t}} \qquad \text{(Eq. 32)}$$

In the common case there is much more solid drug present than necessary to saturate the external phase of the vehicle, i. e., $C >> C_s$, and the above equations may be further simplified to

$$Q = \sqrt{2C \cdot D \cdot C_s \cdot t} \qquad \text{(Eq. 33)}$$

and

$$\frac{dQ}{dt} = \sqrt{\frac{C \cdot D \cdot C_s}{2t}} \qquad \text{(Eq. 34)}$$

If we substitute activities for concentrations we have

$$Q = \sqrt{2a \cdot D \cdot a_s t} \qquad \text{(Eq. 35)}$$

and

$$\frac{dQ}{dt} = \sqrt{\frac{a \cdot D \cdot a_s}{2t}} \qquad \text{(Eq. 36)}$$

where a = the thermodynamic activity of the drug in the vehicle, a_s = the thermodynamic activity of the drug in the external phase of the vehicle, and the other symbols have the significance indicated above.

Higuchi (174) pointed out that the instantaneous rate of release of medicament from the vehicle (equivalent to rate of absorption with opposite sign) may be regulated by controlling a, D, and a_s. If an aqueous solution is the external phase of the vehicle, a_s can be varied by changing the effective pH of the vehicle for poorly soluble weakly acidic and basic drugs. The activity coefficient of the molecular form of such drugs (the molecular or unionized form being assumed to be the species absorbed) is a rapidly changing function of pH for pH values greater than the pKa for acidic drugs and less than pKw − pKb for weakly basic drugs. For an acidic drug we have

$$HA \rightleftharpoons H^+ + A^- \qquad \text{(Eq. 37)}$$

$$K_a = \frac{a_{A^-} \cdot a_{H^+}}{a_{HA}} \qquad \text{(Eq. 38)}$$

$$a_{HA} = \frac{a_{A^-} \cdot a_{H^+}}{K_a} = \frac{a_{A^-} \cdot 10^{-pH}}{K_a} = \frac{a_{A^-}}{K_a \cdot 10^{pH}} \qquad \text{(Eq. 39)}$$

As the pH of the vehicle increases, the activity of the molecular species, a_{HA}, decreases rapidly in the region where pH > pK_a or $10^{pH} > K_a$.

For basic drugs we have

$$B + H_2O \rightleftharpoons BH^+ + OH^- \qquad \text{(Eq. 40)}$$

$$K_b = \frac{a_{BH^+} \cdot a_{HO^-}}{a_B} = \frac{a_{BH^+} \cdot a_{H_2O}}{a_B \cdot a_{H^+}} \qquad \text{(Eq. 41)}$$

$$a_B = \frac{a_{BH^+} \cdot a_{H_2O}}{K_b \cdot a_{H^+}} = \frac{a_{BH^+} \cdot 10^{-pK_w}}{K_b \cdot 10^{-pH}} = \frac{a_{BH^+} \cdot 10^{pH}}{K_b \cdot 10^{pK_w}} \qquad \text{(Eq. 42)}$$

As the pH of the vehicle increases, the activity of the molecular species, a_B, increases rapidly in the region where pH > pKb or pH > pKw − pKa.

Hence, in the pH ranges discussed above, the activity of the molecular species of a weakly acidic drug will be higher at lower pH values and the activity of molecular species of a weakly basic drug will be higher at higher pH values (174). Changing the pH at which the vehicle is buffered by two pH units can make a hundredfold difference in the activity of the molecular species in the vehicle.

Since the instantaneous rate of absorption, dQ/dt, is proportional to the square root of the activity of the molecular species, then changing the vehicle pH by two pH units can make a ten-times difference in instantaneous rate of absorption.

Considering the above equations it is obvious that if one molecule of the molecular species is absorbed (lost from the ointment), another molecule must be formed from A^- or BH^+ in order to keep K_a or K_b constant. Not taking this into consideration has led to some obvious errors in reasoning. For example, Stolar, Rossi, and Barr (175) stated: "It is doubtful that the concentration of free salicylic acid present at such pH values (6.95% sodium salicylate in hydrophilic ointment with continuous aqueous phase having a pH of 6.2 to 6.5) could account for the amount detected in the blood of rabbits examined during this investigation." Since salicylic acid has a pK_a of 2.97, there is a very low activity of salicylic acid in the external phase of their preparation. However, as indicated above, once the molecules of salicylic acid which are available are absorbed more are formed from the salicylate ions. In the same paper (175) these authors gave blood level data for absorption of salicylic acid from an ointment containing 6% salicylic acid in the same vehicle, hydrophilic ointment. The initial slope of the blood level curve resulting from the latter ointment is considerably steeper than the initial slope of the blood level curve resulting from application of the equivalent ointment containing sodium salicylate. This difference in initial slopes is indicative that the rate of absorption was considerably faster from the ointment containing salicylic acid than from the ointment containing sodium salicylate, which agrees with the predictions of Higuchi (174) above.

It was indicated previously that the activity of the drug in the vehicle may also be increased by using a different crystalline modification of the solid phase of the drug, particularly where more complex organic drugs are involved. Such different crystalline modifications have different thermodynamic activities at room temperature. More energetic species, however, are metastable, tending to revert to the more stable, less energetic species (174). Hence, the "trick" in such cases would be to use a more energetic species which would have a suitable stability for the expected life of the product. These are highly specific situations for a particular drug in a particular vehicle and are usually not predictable by common knowledge but require extensive experimentation.

For a given concentration of drug in certain vehicles, the activity coefficient of the drug, and consequently the thermodynamic activity of the drug in the vehicle at that concentration, may vary by a factor as much as a thousandfold (174) from one vehicle to the next. Solutes held firmly by the vehicle, such as when the drug forms a soluble complex with the vehicle, exhibit low activity coefficients; hence, the rate of release from such drug-vehicle combinations will be slow. Solutes held "loosely" by the vehicle (less affinity of the vehicle for the drug or solute) exhibit high activity coefficients; therefore, the rate of release from such drug-vehicle combinations will be faster (174). This helps to explain some of the investigations which have involved use of salicylic acid (175–179) and sulfanilamide (178, 180) as tracers in dermatologic research. The activity coefficients of these tracers and their thermodynamic activities at a given concentration in the various vehicles would be expected to vary widely just on the basis of the nature of the vehicles and tracers employed in these studies. Polyethylene glycols complex salicylic acid; therefore, one would expect the thermodynamic activity of salicylic acid at a given concentration in polyethylene glycol ointment to be very low. The results obtained by Shelmire (177), Stolar, *et al.* (175, 176), Plein and Plein (178), and Nogami and Hanano (179 *A*) using bases containing polyethylene glycols and Carbowaxes are explicable on the above basis. The very "flat" blood level curve of salicylic acid, parallel but only slightly above the time axis, which Stolar, *et al.* (175), obtained when salicylic acid in polyethylene glycol ointment was applied to rabbit's skin is indicative of the low thermodynamic activity of salicylic acid in this vehicle. Also, it shows that the complex acts as a reservoir releasing drug very slowly but at an essentially constant rate to the skin barrier. Hydrophilic ointment contains 37% w/w water and one would expect the thermodynamic activity of salicylic acid in this vehicle to be quite high. This would be also true for the water-containing emulsions used by Shelmire (177). The rate of penetration of salicylic acid from these vehicles was reported to be very rapid (175, 177). The thermodynamic activities of salicylic acid at a given concentration in hydrophilic petrolatum and petrolatum would be expected to be intermediate between those in hydrophilic ointment and polyethylene glycol ointment on the basis of solubilities and the anhydrous nature of the former two ointments. If one takes into consideration that the area under the blood level-time plot is a measure of the amount of salicylic acid absorbed (8) and with a given tracer the initial slope of the blood level curve is indicative of the rate of absorption, then the above considerations appear to explain largely the results of Stolar, Rossi, and Barr (175, 176). Similarly, the thermodynamic activity of sulfanilamide at a given concentration in various ointment bases would vary widely and should be taken into consideration in considering the results obtained by Plein and Plein (178) and Gemmell and Morrison (180).

The diffusion coefficient of the drug in the vehicle is an important consideration for the pharmacist also. Higuchi (174) pointed out that the diffusion coefficient, D, is inversely proportional to the viscosity of the vehicle. This follows from Stokes-Einstein equation for the diffusion of colloidal particles, namely

$$D = \frac{RT}{6\pi r\eta N} \qquad \text{(Eq. 43)}$$

where D is the diffusion coefficient, R is the gas constant, T is the absolute temperature, r is the mean radius of the particles, η is the viscosity, and N is the Avagadro number. If it is assumed that all the particles have the same size, that they are spherical in shape, and that their density is the same as for the disperse phase in bulk, then the relationship

$$r = \sqrt[3]{\frac{3m}{4\pi\eta\rho}} \qquad \text{(Eq. 44)}$$

can be derived where m is the mass of n particles present in volume V with a density of ρ. Hence, D also varies inversely approximately as the cube root of the molecular weight; thus, for very large, complex organic drugs the molecular weight may be an important factor to take into consideration. W. I. Higuchi, *et al.* (29), indicated diffusion coefficients may vary with the different ionic and nonionic species present, with the concentration, and with the thickness of the diffusion barrier. Different salts of an acidic or basic drug will have different diffusion coefficients than each other and all may be different than the nonionized species in a given vehicle. Similarly, different derivatives and modifications of one drug moiety may be expected to have different diffusion coefficients in a given vehicle.

The diffusion coefficient of sodium salicylate in the anhydrous bases, hydrophilic petrolatum and petrolatum, would be expected to be lower than the diffusion coefficient of salicylic acid in the same vehicles. Also, the solubility, C_s, of sodium salicylate in these same anhydrous "oily" vehicles would be much less than the solubility of salicylic acid in these vehicles. These considerations further aid in explaining the results of Stolar, Rossi, and Barr (175).

The rate of solution, in say, mg./hour, is directly proportional to the surface area available, as we have shown previously. In an ointment or cream containing suspended solid the total surface area, the median particle size diameter, and the particle size distribution may be very important and should be considered as important variables in such studies.

Absorption from Solution.—If a drug is initially uniformly dissolved in a homogeneous vehicle it can be shown that the amount of material absorbed per unit area, Q, from the applied phase is given by (174, 181)

$$Q = h \cdot C_0 \left[1 - \frac{8}{\pi^2} \sum_{m=0}^{\infty} \frac{1}{(2m+1)^2} \exp\cdot \left\{ \frac{}{} - \frac{D(2m+1)^2 \cdot \pi^2 \cdot t}{h^2} \right\} \right] \qquad \text{(Eq. 45)}$$

where h = the thickness of the applied phase, C_0 = the initial concentration of the drug, D = the diffusion constant of the drug in the vehicle, m = an integer, and t = the time elapsed since initial application.

Higuchi (174) stated that this will rarely apply to percutaneous absorption through intact skin since the diffusion coefficient of any drug readily taken in through such a barrier will be so great as to maintain a uniform concentration in the applied phase.

Hanano(179*B*) studied the absorption of salicylic and benzoic acids from aqueous and buffer solutions through intact human skin. The equations he derived, which are shown below, would be valid when the rate of absorption is limited by either drug clearance below the barrier layer or by passage through the barrier layer.

$$\frac{dC}{dt} = -\frac{AC}{RV} = -\frac{A(1-a)}{RV} \qquad \text{(Eq. 46)}$$

This integrates, with $c = 0$ at $t = 0$ to

$$C = C_0 \exp\cdot \frac{-A(1-a)t}{RV} \qquad \text{(Eq. 47)}$$

where C_0 = the initial concentration of the drug in the vehicle, C = the concentration of the drug in the vehicle at time t, R = the overall resistance of the skin barrier, V = the volume of the vehicle, A = the area to which the solution was applied, and a = the degree of dissociation of the weak acid.

From the above one can obtain

$$t = \left[ln \left(\frac{C_0}{C_0 - C} \right) \right] \left[\frac{RV}{A(1-a)} \right] \qquad \text{(Eq. 48)}$$

Hence, the time necessary to reduce the concentration of drug from C_0 to C, i. e., the time necessary to absorb $V(C_0 - C)$, is inversely related to the fraction of unionized molecules, namely $1 - a$, in the solution applied. This agrees with some of the discussion before concerning relationships between pH, pKa, and rate of absorption of acidic drugs.

Organic solvents alter skin resistance towards penetration. This phenomenon is possibly caused by marked changes produced by such solvents in the activity coefficient and diffusion coefficient of the drug in the skin barrier (174). Shelmire (173) reviewed some literature which indicated that ether extraction of isolated epidermis greatly increases the rate of water diffusion; therefore, water is lost faster from the stratum corneum. This would imply that ether-extraction of skin alters the activity of water in the skin barrier phase.

Additional Literature on Percutaneous Absorption.—Rothman (182) has an excellent review of the literature to 1954. Gemmell and Morrison (183) made a fine review principally concerned with the various methods applied by various investigators to evaluate dermatologic preparations both *in vitro* and *in vivo*. Since the latter review Lueck, *et al.* (184), reported a method suitable for measuring the permeation of continuous ointment films by diffusional processes when the penetrating agent is in the vapor state. It was shown that in both liquid contact and gaseous contact the permeation constant was related directly with the distribution coefficient and inversely with the thickness of the film. The studies indicated that the selection of a given semisolid vehicle for a given drug should be guided primarily by the relative solubility of the drug in the vehicle. The reviewer feels sure these authors intended a_s in place of solubility. One could have a high solubility due to complex formation but in such a case a_s would be very low and, thus, the ointment would be a poor one.

Various comparative studies where one or more drugs or tracers were incorporated into one or more vehicles have been reported (185–192). As stated earlier, this review is not intended to cite all such references.

The possible need for toxicity testing of dermatologic preparations was recently indicated by Snyder (193). The real problem is to determine what levels of exposure can be considered safe since almost any drug will probably penetrate the skin to some extent and there is no sharp dividing line between toxic and nontoxic compound (193). Adriani

and Campbell (194) showed how important this problem can become by investigating the blood levels of tetracaine, a local anesthetic, following rapid intravenous administration, slow intravenous infusion, subcutaneous infiltration, and topical application using several vehicles.

During the research and development leading up to a marketable dermatological preparation, comparative absorption tests are often performed using animals. There may be a considerable difference in the rate and extent of absorption of a given agent between animals and man. For example, Rubin (195) recently reviewed the literature on the percutaneous absorption of vitamins. He reported that vitamin A is absorbed through animal skin relatively rapidly but is absorbed in man to only a relatively small degree. On the other hand, panthenol and pyridoxine appear to penetrate both animal and human skin relatively well (195).

PARENTERAL ABSORPTION

A parenteral preparation is one which is intended for administration through or under one or more layers of skin (196). There are many types of injection sites, including subcutaneous, intramuscular, intravenous, intradermal, hypodermal, intra-arterial, intrapleural, intraperitoneal, intra-articular, intracardial, intraspinal, and intracerebral (197). There appears to be much less known about absorption from these various sites of administration than is known about absorption from the gastrointestinal tract or about percutaneous absorption. Despite the fact that the early testing of drugs in animals is mainly by parenteral routes of administration, and that many millions of dollars worth of parenteral products are sold annually for use in man, there has been very little good research done on the factors effecting absorption of drugs from parenteral sites of administration. During the past twenty years there has been considerable patent activity in prolonged-action parenteral formulations. These are formulations whose intended purpose is to control the rate of liberation of a drug from the "depot" or "pool" at the site of injection. No attempt will be made to cover these patents in this review.[3]

Absorption is not involved when a drug is administered parenterally by the intravenous, intra-arterial, intraspinal, and intracerebral routes. However, when the drug is administered by the subcutaneous, intramuscular, intradermal, hypodermal, intra-articular, intrapleural, or intraperitoneal routes then a depot of some type is formed and the drug must leave the depot and reach the blood or lymph systems by some process or processes.

Teorell (3) in 1937 discussed the theoretical aspects of the kinetics of distribution of drugs administered intravenously and intra-arterially. He derived formulas which aid estimation of the effects of various variables when the drug is administered by rapid injection and by continuous infusion. Some simple rules were derived from the equations which apparently are not taught in the usual advanced pharmacology courses. He also discussed the influence of the rate of injection and the magnitude of the dose in regard to the pharmacological effect produced. Important consequences have to be considered when dealing with substances which are quickly destroyed or otherwise rapidly disappear from the volume of distribution. The dependence of the maximum amount of a drug which can accumulate in the volume of distribution on the half-life of the particular drug was discussed by Boxer (6) and others. However, it still appears to be largely unknown by many in the biological sciences that one can calculate the accumulation residue if the dosage regimen and the half-life of the drug are known. Indeed, determination of the half-life of a drug in a suitable sample of patients or subjects is the only rational way to arrive at proper dosage regimens, as has been pointed out in the literature (6, 7, 11, 12).

When discussing the extravascular routes of administration, Teorell (3) assumed that the drug would disappear from the depot according to first-order kinetics, i. e., the rate of release from the depot is proportional to the amount remaining in the depot. Hence, the rate of absorption from a parenteral depot would be most rapid immediately following the injection and the rate would slow down with increase in time after administration. A plot of the logarithm of the amount of drug remaining in the depot *versus* time after administration would yield a straight line if the rate of absorption from the depot was pseudo first order. Some authors (198–201) have produced data to test this hypothesis but failed to plot the data in this way. Had they done so they would have learned much more from their data. For example, they could have compared the rate constants when certain variables such as volume of fluid injected, concentration of drug in the vehicle, etc., were varied; they could have estimated the time when a certain per cent, say 90%, of the drug would be released from the depot; they would have been able to calculate dosage regimens for use of the drug. The data of these authors and others certainly appear as if Teorell's hypothesis was correct, i. e., in many cases drugs do appear to disappear from parenteral depots according to pseudo first-order rates. In the case of implantation, however, pseudo zero-order rates, or constant rates of release from the depots are usually observed (202). This would be expected if the surface area of the implant is restricted and constant and, hence, dissolution from the solid is rate-determining.

From a pharmaceutical viewpoint one is principally interested in what factors we may alter which contribute to the rate of release from a parenteral depot. In some cases we may be interested in making the drug more rapidly available to the fluids of distribution after administration, and in other cases we may wish to markedly slow the rate to produce a sustained or prolonged action injectable preparation.

Pharmaceutical factors which may influence the rate of release of drug from a parenteral depot: (*a*) The volume of the injected formulation (201). (*b*) The concentration of drug in the vehicle in th

[3] "A Supplementary Bibliography on Parenteral Absorption and Formulation of Parenteral Products," containing ninety-two recent nonpatent references and seventy-two recent patent references has been prepared. The bibliography contains the authors' names, the titles of the articles, and the journal or patent references. Copies may be obtained from the author upon request.

case of solutions (201) and the solids/vehicle ratio in the case of suspensions (203). (*c*) The presence or absence of enzymes such as hyaluronidase in the formulation (201, 202). (*d*) The surface area of the depot (202, 203) and of the drug. (*e*) The nature of the solvent—whether aqueous, nonaqueous, or a mixture of water and a nonaqueous solvent or solvents (202, 203). (*f*) The tonicity of the formulation (202). (*g*) The visocity of the vehicle (202, 203). (*h*) The rate of dissolution of the drug if suspended in the vehicle, or the rate of dissolution of some form of the drug if this form is precipitated in the depot. Since the rate of dissolution is largely influenced by the solubility of the drug in the fluids at the site of injection then the solubility of the drug is important (202, 203). Both the solubility and the rate of dissolution are influenced by derivitization of the drug, by salt formation, and by complex formation (202, 203). (*i*) The crystal form of the drug used (203). (*j*) The particle size and particle size distribution of the drug in suspension in the vehicle (203). (*k*) Coating of the particles of drug to delay absorption or decrease release rate (203). (*l*) The presence or absence of adjuvants such as suspending agents (202). (*m*) The presence or absence of vasoconstrictors (202). (*n*) The partition coefficient of the drug between the vehicle and the tissue fluid if the drug is injected in solution in an oil or oil-like vehicle (202). (*o*) The chemical nature of the drug, i. e., whether it is a neutral molecule, a weak acid, a weak base, or a salt.

One may generally summarize the above factors by saying that they are the same factors which control the rate of dissolution of a solid and/or its transfer from one phase to another. There are many lifetimes of useful research in just using the list above as a guide and designing experiments in such a way that one isolates only one variable at a time and studies its relative importance with typical types of drugs such as neutral molecules, weak acids, weak bases, and their salts. If we had such fundamental information the formulation of a given parenteral product would not be quite so empirical as it is at present.

In man, purely local reactions to injected material may take the form of pain occurring immediately upon injection of the material or very shortly thereafter, or of inflammatory reactions, sometimes with abscess formation occurring during the days following the injection. More rarely, reactions remote in time from the injection may occur, for example, the paraffin granuloma. Trial in man is often the only way to determine if a local reaction to an injection will occur (204). Animal tests are useful in predicting whether a local reaction may occur in man (204, 205). The mechanism of pain occurring after injection and why some drugs cause more pain than others seem to be very poorly understood. This seems to be a fruitful area of research which to the reviewer's knowledge has not been approached. To what extent such local reactions influence the rate of absorption from a parenteral depot is little known also. The recent work of Schou (206–208) indicates that liberation of histamine at the site of injection, due to trauma, decreases the rate of absorption, particularly immediately after injection.

EPILOGUE

James B. Conant (209) wrote: "As a first approximation, we may say that science emerges from the other progressive activities of man to the extent that new concepts arise from experiments and observations, and the new concepts in turn lead to further experiments and observations. . . . The texture of modern science is the result of the interweaving of the fruitful concepts. The test of a new idea is therefore not only its success in correlating the then-known facts but much more its success or failure in stimulating further experimentation or observation which in turn is fruitful. This *dynamic quality of science*[4] viewed not as a practical undertaking but *as a development of conceptual schemes*[4] seems to me to be close to the heart of the best definition." Hence, it would appear that one of the major objectives of research, in general, should be to evolve new conceptual schemes. These may be modified or displaced in the future, yet meanwhile, they have organized knowledge, effected progress, and stimulated future research.

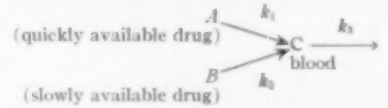
These general quotations and remarks apply to the specific subject matter of this review, namely absorption. We need more of the type of research that leads to new *concepts* and less of that which leads to new *things*. This is particularly true in the role which pharmacy will play in the future. We need better control of variables and better planning of the experiments. Quite often an hour spent in meditating on what one is going to do is worth more than a year in a laboratory. We need better planning of experiments in which blood and urine levels of drug are determined and mathematical analysis of the results.

Perhaps in the future we can relate the physical and chemical properties of different drugs and their dosage forms to their absorption patterns *in vivo*. Such research would involve very carefully controlled *in vitro* and *in vivo* measurements followed by proper correlation of results. Should this become a reality we may be able to make quite accurate predictions of the rate and extent of absorption of an entirely new compound in a certain dosage form on the basis of the physical and chemical properties.

APPENDIX

Models, differential equations, solutions of the differential equations, and rate constants used in preparing the plots shown in Fig. 2

Model I.—A hypothetical model for cases where there is absorption of the drug from both the stomach and the intestines and/or where a sustained action dosage form contains some readily available drug (in compartment *A*) and some slowly available drug which is released at a constant rate.



D excretion and metabolic products

[4] Italics added.

Assumptions.—(*a*) The rate constants k_1 and k_2, are first-order rate constants, in hours^{-1}, and $k_1 \neq k_2$. (*b*) The rate constant, k_0, is a zero-order rate constant in units of fraction of total amount per hour. (*c*) Assume $X_A = 0.3$ at $t = 0$; $X_B = 0.7$ at $t = 0$; $X_C = X_D = 0$ at $t = 0$; and $t \leq 0.7/k_0$. (*d*) Release from the dosage form is rate-determining the absorption process.

If X_A, X_B, X_C, X_D are the amounts in compartments A, B, C, and D, respectively, at time t, then

$$\frac{dX_A}{dt} = -k_1X_A \text{ and } X_A = 0.3 \exp{\cdot -k_1t}$$

$$\frac{dX_B}{dt} = -k_0 \text{ and } X_B = 0.7 - k_0t$$

$$\frac{dX_C}{dt} = k_0 + k_1X_A - k_2X_C \text{ and } X_C = k_2 X_C$$

$$\frac{0.3k_1}{k_2 - k_1}\,[\exp{\cdot -k_1t} - \exp{\cdot -k_2t}] + \frac{k_0}{k_2}\,[1 - \exp{\cdot -k_2t}]$$

$$\frac{dX_D}{dt} = -\frac{dX_A}{dt} - \frac{dX_B}{dt} - \frac{dX_C}{dt} = k_3 X_C$$

$$X_D = 1 - X_A - X_B - X_C$$

The rate constants used were as follows:

Set	k_1 (hr.$^{-1}$)	k_0 (hr.$^{-1}$)	k_2 (hr.$^{-1}$)
1	1.0	0.14 (i. e., 5 hr. to release the 0.7)	0.1155
2	1.0	0.0325 (i. e., 21.5 hr. to release the 0.7)	0.1155
3	0.5	0.0325 (*ibid.*)	0.1155

Model II.—A hypothetical model for cases where absorption from the stomach is negligible but the drug is absorbed in the intestines. A sustained action dosage form releases drug at a constant rate in the intestines. Stomach emptying is assumed to be a first order process. The dosage form contains no quickly available drug.

$$A \xrightarrow{k_1} B \xrightarrow{k_0} C \xrightarrow{k_2} D$$

stomach — intestine — blood — excretion + metabolic products

Assumptions.—(*a*) The rate constants, k_1 and k_2, are first-order rate constants, in hours^{-1}, and $k_1 \neq k_2$. (*b*) The rate constant, k_0, is a zero-order rate constant, in units of fraction of total amount per hour. (*c*) Assume $X_A = 1$ at $t = 0$ and $X_B = X_C = X_D = 0$ at $t = 0$. (*d*) Release from the dosage form is rate-determining the absorption process. (*e*) The solutions to the differential equations apply only to certain values of t.

If X_A, X_B, X_C, and X_D are the amounts in compartments A, B, C, and D, respectively, then

$$\frac{dX_A}{dt} = -k_1X_A \text{ and } X_A = \exp{\cdot -k_1t}$$

$$\frac{dX_B}{dt} = k_1X_A - k_0 \text{ and } X_B = 1 - \exp{\cdot -k_1t} - k_0t$$

$$\frac{dX_C}{dt} = k_0 - k_2X_C \text{ and } X_C = \frac{k_0}{k_2}\,(1 - \exp{\cdot -k_2t})$$

$$\frac{dX_D}{dt} = -\frac{dX_A}{dt} - \frac{dX_B}{dt} - \frac{dX_C}{dt} = k_2X_C \text{ and}$$

$$X_D = k_0t - \frac{k_0}{k_2}\,(1 - \exp{\cdot -k_2t})$$

The rate constants used were as follows:

Set	k_1 (hr.$^{-1}$)	k_0 (hr.$^{-1}$)	k_2 (hr.$^{-1}$)
1	1.0	0.20 (i. e., 5 hr. to release the total amount, 1)	0.1155
2	1.0	0.05 (i. e., 20 hr. to release the total amount, 1)	0.1155
3	0.5	0.05 (*ibid.*)	0.1155

Model III.—Similar to model II except assumed that drug is released from the dosage form at a first-order rate rather than at a zero-order rate as in model II. All three constants, k_1, k_2, and k_3 are then first-order rate constants, in hours^{-1}.

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D$$

stomach — intestine — blood — excretion + metabolic products

$$\frac{dX_A}{dt} = -k_1X_A \text{ and } X_A = \exp{\cdot -k_1t}$$

$$\frac{dX_B}{dt} = k_1X_A - k_2X_B \text{ and}$$

$$X_B = \frac{k_1}{k_2 - k_1}\,(\exp{\cdot -k_1t} - \exp{\cdot -k_2t})$$

$$\frac{dX_C}{dt} = k_2X_B - k_3X_C \text{ and } X_C =$$

$$k_1k_2\left[\frac{\exp{\cdot -k_1t}}{(k_2 - k_1)(k_3 - k_1)} + \frac{\exp{\cdot -k_2t}}{(k_1 - k_2)(k_3 - k_2)} + \frac{\exp{\cdot -k_3t}}{(k_1 - k_3)(k_2 - k_3)}\right]$$

$$\frac{dX_D}{dt} = -\frac{dX_A}{dt} - \frac{dX_B}{dt} - \frac{dX_C}{dt} = k_3X_C \text{ and}$$

$$X_D = 1 - X_A - X_B - X_C$$

The rate constants used were as follows:

Set	k_1 (hr.$^{-1}$)	k_2 (hr.$^{-1}$)	k_3 (hr.$^{-1}$)
1	1.0	0.5	0.1155
2	1.0	0.1	0.1155
3	0.5	0.1	0.1155

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Comparison of Dissolution and Absorption Rates of Different Commercial Aspirin Tablets

By GERHARD LEVY

The absorption rates of several types of commercial aspirin tablets have been determined by the urinary excretion method. The results indicate that the *in vivo* absorption rate is proportional to *in vitro* dissolution rate determined by a method previously described (1). An explanation is offered for some of the apparently conflicting results obtained by different clinical investigators who have studied the absorption rates of different types of aspirin. Some important but often neglected variables related to the clinical evaluation of aspirin absorption are discussed. It is proposed that the U. S. P. tablet disintegration test be replaced by a dissolution test.

IN A RECENT study of factors affecting the absorption rate and gastrointestinal irritant effect of aspirin (1) it was concluded that (*a*) absorption rates and incidence and severity of local irritation are interrelated, (*b*) both of these characteristics are a function of the dissolution rate of aspirin in its particular dosage form, and (*c*) there are significant differences in *in vitro* dissolution rates among different nationally distributed brands of aspirin tablets.

The purpose of the investigation presented here was to study the relation between the *in vitro* dissolution rate and the gastrointestinal absorption rate of commercial brands of aspirin tablets. Some important but often neglected variables related to the comparative clinical evaluation of aspirin absorption rates will also be outlined and discussed in this report.

There are three prime reasons that make a study of this nature important. First, considerable gastrointestinal hemorrhage may occasionally be caused by aspirin particles adhering to the mucosa. This has been shown by several investigators who have made qualitative (2–4) and quantitative (5–7) determinations of gastrointestinal blood loss as a consequence of aspirin ingestion. While the general safety of aspirin has been demonstrated by its widespread and prolonged use, even a low percentage of untoward side effects from this drug assumes considerable importance due to the large quantities that are used by the population. It has been estimated (8) that the production of aspirin in 1959 was equivalent to about 60 million five-grain tablets per day.

A second reason for the choice of aspirin as the drug to be studied is based on the fact that it is rapidly absorbed from all parts of the gastrointestinal tract, including the stomach. Thus it can serve as an excellent "marker" to assess the effect of certain formulation and dosage form characteristics upon absorption rate. It would seem likely that tablet formulation properties which cause delayed biological availability would be most readily recognizable with drugs that are by nature rapidly absorbed from the gastrointestinal tract.

Thirdly, it is desirable to develop *in vitro* methods which can reflect the biological availability of therapeutic agents. The elucidation of an *in vitro* method which can serve to predict the rate of absorption of aspirin in tablet form could be a first step toward the development of similar *in vitro* methods for compressed tablets of other drugs, especially weak acids.

PHYSIOLOGICAL CONSIDERATIONS

It has been shown that aspirin is readily absorbed from the stomach of man (9, 10). While initial absorption of aspirin takes place across the gastric mucous membrane, the major site of absorption appears to be the proximal portion of the small intestine (11). This is so because of the much greater absorptive surface that presents itself to the drug in the intestine, as compared to the stomach. In addition, consideration must be given to the difference in environmental factors present in the stomach and the intestinal tract. Intestinal fluids, by reason of their higher pH, would bring about much faster dissolution of aspirin than gastric fluids. It can also be assumed that much greater agitative forces are brought to bear upon aspirin solids in the intes-

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tine due to the greater proximity of peristaltic waves by reason of the small intestinal lumen. This would also favor rapid dissolution of the drug. The difference in the rate of aspirin absorption from the stomach and the intestine are effectively demonstrated by Weikel and Lish on rats (11). At no time were these workers able to recover drug from the intestine, but they recovered aspirin from the stomach of their test animals thirty, ninety, and one hundred and eighty minutes after oral administration.

Recently, Moore, *et al.* (12), found that faster stomach emptying increased the toxicity and shortened the onset of action of sodium salicylate. They too credited this effect to the more favorable conditions for absorption found in the intestine.

The clinical reports of mucosal irritation or erosion due to aspirin generally refer to the gastric mucosa; this is in accord with the reasoning presented above. Higher pH and more efficient absorption would result in rapid dissolution and removal of the potentially irritating drug particles from the intestine. Conversely, the greatest chance for irritation and erosion is found in the stomach, where conditions could cause solid aspirin particles to persist in contact with the mucous lining for a considerable time.

Since the initial absorption of aspirin occurs form the stomach, and since the rate of this absorption is proportional to the amount of aspirin dissolved in the gastric fluids, the *in vivo* dissolution rate of aspirin in tablet form in the stomach would be reflected by the initial absorption rate. The relatively unfavorable conditions for dissolution in the stomach would accentuate the differences in the dissolution characteristics of various brands of aspirin tablets.

On the other hand, conditions in the intestinal tract are so favorable to rapid dissolution that product-to-product differences should tend to disappear. Unless the tablets are enteric coated, of the sustained-release type, or are extremely resistant to disintegration, they should all dissolve quite rapidly.

The blood level picture after aspirin administration should then be as follows: variations in salicylate plasma levels due to different absorption rates caused in turn by product-to-product differences in dissolution rate would be apparent in the initial stages of the absorption process. These differences would largely disappear in subsequent stages of the absorption process. This in fact is the common clinical picture; wherever differences in salicylate plasma levels can be demonstrated, they are found during the first twenty or thirty minutes after drug administration and usually disappear later on. It remains to be explained why these differences in blood levels, which are apparent for only a short time, should be of any clinical significance or interest. Many disparaging comments that have so undeservedly been directed against studies dealing with a comparative evaluation of aspirin absorption are due to a lack of recognition of the major significance of differences in initial absorption rate. It is argued that aspirin is not an emergency drug and that small differences in onset of its therapeutic effect are generally not very important. These initial differences do, however, reflect the rapidity or slowness of dissolution of a given dosage form in the stomach and, therefore, the probability that a product may cause gastrointestinal damage.

EXPERIMENTAL

Test Products. The products tested consisted of three brands of plain aspirin tablets, one brand of buffered aspirin, and one brand of calcium acetylsalicylate. The brands, their dissolution characteristics, and their disintegration times are listed in Table I.

TABLE I.—DISSOLUTION AND DISINTEGRATION CHARACTERISTICS OF ASPIRIN TABLETS (0.3 GM.) USED IN THE ABSORPTION STUDIES

Product	Amount in Solution at 10 min.,[a] mg.	Standard Deviation, mg.	Disintegration Time,[b] sec.	Description of Product
A	242.2	33.9	256	Calcium acetylsalicylate
B	205.0	27.5	35	Buffered drug with aluminum glycinate and magnesium carbonate
C	165.1	19.0	<10	Plain drug
D	157.6	36.2	13	Plain drug
E	126.8	20.0	<10	Plain drug

[a] Average of 6 tablets. [b] U. S. P. XV method (with disks) in 0.1 *N* hydrochloric acid.

Dissolution Procedure. The dissolution rate of the various brands was determined in 0.1 *N* hydrochloric acid at 37° under standardized agitation conditions. Details of the procedure have been reported previously (1).

Absorption Tests. Twenty-four healthy adult males served as test subjects. Their weights and ages are listed in Tables II and III. Fourteen of the subjects made up group A and the other ten constituted group B. Absorption tests were initiated in the morning on empty stomach. Both studies were of cross-over type and Latin square design.

The protocol for group A was as follows: (*a*) Subjects emptied their bladder of overnight urine and ingested one glass of water. (*b*) About one hour later, subjects collected urine blanks and swallowed two 0.3-Gm. aspirin tablets whole, followed by exactly 100 ml. of water. (*c*) Urine samples were collected one, three, five, seven, and nine hours after drug ingestion.

The protocol for group B was as follows: (*a*) As above. (*b*) Exactly thirty minutes later they collected a urine blank and then swallowed two 0.3-Gm. aspirin tablets whole, followed by exactly 200 ml. of water. (*c*) Thirty and sixty minutes later, urine samples were collected.

All subjects were cautioned not to eat for two hours after the initiation of the test. The tests were carried out seven days apart, with two exceptions in which tests were three days apart. Subjects were cautioned not to ingest any form of salicylate for forty-eight hours prior to the test.

Analytical Method.—Salicylate in the urine was determined colorimetrically with ferric nitrate reagent (13). This reagent develops a purple-colored complex with free salicylate and salicyluric acid, but not with glycuronides of salicylic acid unless these are first hydrolyzed (14). The method is considered adequate and useful for comparative

TABLE II.—CUMULATIVE APPARENT SALICYLATE EXCRETED FOLLOWING ADMINISTRATION OF TWO 0.3-GM. ASPIRIN TABLETS, MG.

Subject	Age	Wt	Product B					Product D					Product E				
			1 hr.	3 hr.	5 hr.	7 hr.	9 hr.	1 hr.	3 hr.	5 hr.	7 hr.	9 hr.	1 hr.	3 hr.	5 hr.	7 hr.	9 hr.
A	22	155	22.8	84.7	132	190	232	28.3	110	172	240	290	13.0	91.7	160	194	248
B	20	190	16.3	86.3	164	232	285	8.67	66.3	136	201	246	13.7	90.1	178	227	278
C	19	175	19.8	111	163	215	265	17.7	98.0	176	254	315	15.9	101	204	277	329
D	26	166	20.8	73.0	109	131	151	10.4	44.4	75.0	112	146	4.55	60.2	128	202	249
E	19	156	34.0	134	234	329	388	1.50	81.0	161	239	295	9.74	84.0	188	262	327
F	20	145	18.5	80.2	160	205	247	13.8	84.5	148	188	223	9.41	49.7	105	147	..
G	24	180	11.7	97.3	168	243	282	24.1	117	228	250	309	17.1	92.4	205	280	302
H	22	155	18.6	90.7	171	234	285	8.10	66.1	148	215	272	11.8	77.2	146	209	262
I	27	175	12.0	86.0	139	190	234	15.1	108	193	259	..	16.6	112	201	269	333
J	19	160	24.5	112	171	252	305	18.0	92.6	194	260	334	15.2	88.6	190	271	335
K	19	175	17.8	90.3	176	243	291	25.2	132	258	313	362	11.8	76.5	152	225	283
L	24	150	6.11	63.7	143	196	212	7.36	73.2	134	173	252	4.23	59.1	125	182	240
M	19	180	28.4	117	216	323	340	8.33	84.3	190	269	318	14.9	92.8	124	181	247
N	20	160	8.25	68.0	71.7	89.7	121	3.98	52.4	99.3	139	172	11.2	58.8	105	144	178
Mean			18.5	90.3	158	220	261	13.6	86.4	165	222	272	12.1	81.0	158	219	278

TABLE III.—CUMULATIVE APPARENT SALICYLATE EXCRETED FOLLOWING ADMINISTRATION OF TWO 0.3-GM. ASPIRIN TABLETS, MG.

Subject	Age	Wt.	Product A		Product C		Product E	
			½ hr.	1 hr.	½ hr.	1 hr.	½ hr.	1 hr.
O	21	153	3.2	18.4	2.2	10.1	2.3	16.9
P	21	157	1.9	11.1	2.0	9.0	3.3	9.8
Q	20	173	2.5	14.8	3.1	16.7	3.2	18.1
R	20	190	5.8	21.6	8.9	26.1	3.0	14.2
S	21	185	4.4	23.6	5.6	16.0	2.8	12.7
T	29	175	4.6	18.4	6.3	19.6	1.3	16.0
U	25	210	12.9	42.2	3.7	18.2	6.5	25.8
V	22	180	6.1	22.4	5.1	19.5	3.8	13.9
W	24	185	3.1	37.0	4.1	27.2	3.0	15.9
X	32	175	10.9	33.8	4.5	18.4	7.8	15.8
Mean			5.5	24.3	4.55	18.1	3.7	15.9

evaluations of salicylate absorption from different dosage forms (15, 16). The absorbance of appropriately diluted samples was determined with the Bausch and Lomb Spectronic 20 colorimeter. These values were properly corrected for blank absorbance.

RESULTS AND DISCUSSION

Table II lists the cumulative amounts of apparent salicylate excreted by the fourteen subjects in group A over a period of nine hours following the ingestion of three different brands of aspirin tablets. It can be seen from the data that product-to-product differences tend to disappear after the first hour.

The results of the experiment supported our prediction that differences between products would be apparent primarily in the initial stages of absorption. For this reason, the second experiment was designed to determine only the initial absorption, as reflected by salicylate excretion thirty and sixty minutes after ingestion of aspirin. The results of this experiment are listed in Table III. The thirty-minute sampling appears to be too premature to reflect adequately the trends that are more readily recognized at the one-hour level.

In Fig. 1, the mean quantities of apparent salicylate excreted one hour after the administration of two 0.3-Gm. aspirin tablets are plotted against their *in vitro* dissolution rate, expressed as the mean amount of aspirin that has gone into solution within ten minutes from an 0.3-Gm. tablet of the various brands tested. The slopes of the two lines fitted to the points by the method of least squares are quite alike. The equation for the group A line is $y = 0.084x + 1.0$ while that of the group B line is $y = 0.074x + 6.2$. Possible reasons for the difference in y intercept will be discussed in a following section.

It is gratifying that despite considerable experimental variation so characteristic of biological processes, the results of the two trials reflect not only the predicted order of absorption, but also essentially the same relation of initial absorption to *in vitro* dissolution rate. This is indicated by the similarity of the two slopes. Significant differences by the "*t*" test could be shown between products A and E ($P < 0.05$) and between products B and E ($P < 0.02$).

Our intent was to show, if possible, the relation of *in vitro* dissolution rate to initial absorption rate of representative commercial preparations. To this end we chose nationally distributed brands of aspirin rather than lesser known products. Some of the latter might possibly have demonstrated more dramatically the differences in initial absorption rate of different brands of generically identical products.

The results also demonstrate the dissolution-rate dependence of aspirin absorption. This has been predicted by Edwards (17) on theoretical grounds. While some of Edwards' reasoning may require modification (18) the essential correctness of his prediction has been shown by Nelson and Schaldemose (16) who compared aspirin absorption rates subsequent to the administration of the drug in

tablets and in solution. The data presented here constitute however, the first comparative determination of aspirin absorption from different *solid* dosage forms that are known to vary in their dissolution characteristics. It would appear that this is a more rigorous demonstration of the solution-rate dependency of aspirin absorption from the gastrointestinal tract of man.

The experimental results point up again the usefulness of the urinary excretion method to study salicylate absorption. This has been demonstrated previously by several workers (15, 16, 19, 20).

It is intriguing to speculate as to the possible reasons for the vertical displacement of the excretion *vs.* dissolution curves in Fig. 1. Had these curves been less similar in slope, one would suspect that the difference is due to normal biological variation between the two groups. There appears to be, however, a more plausible explanation. Group B, represented by the upper curve, ingested a glass of water thirty minutes before taking the drug, and 200 ml. of water with the tablets. Group A, on the other hand, took a glass of water one hour before and only 100 ml. with the aspirin. The greater volume of liquid present in the stomach of the subjects in group B at the start of the experiment may have caused more rapid gastric emptying, either through reflex peristalsis or by dilution of the aspirin, since this drug is known to inhibit the rate of gastric emptying (11). That dilute solutions of salicylate are more rapidly emptied from the stomach than more concentrated solutions representing an equal amount of drug was recently shown in rats (12). It has also been recognized that acceleration of gastric emptying can increase the rate of aspirin absorption (11, 21, 22). In view of the rapid dissolution of aspirin and its rapid absorption from the intestine, it is reasonable to expect that faster gastric emptying would increase absorption from all products almost equally. In this way the one-hour salicylate excretion by subjects of group A could differ by an essentially constant increment from that of group B, accounting for the vertical displacement of the upper curve in Fig. 1. If this hypothesis is borne out by additional investigations, it might be advisable to include the statement "take with a large glass of water" on the label of commercial packages of aspirin tablets.

There are two other points in relation to the data summarized in Fig. 1 that deserve mention. Watson and Pierson (7) determined the gastrointestinal blood loss in 120 subjects taking various forms of salicylate. By means of radio-chromate assay, they found that fecal blood loss increased in the order: salicylate solution < product A < product B ≤ product C. The order of blood loss is identical to the one that could be predicted on the basis of the data summarized in Fig. 1. Furthermore, one could predict by extrapolation of the upper curve in Fig. 1 that salicylate excretion one hour after the ingestion of 650 mg. of aspirin in solution would be 30.8 mg. Nelson (16), who administered this amount of drug in a quantity of water comparable to that used by us for group A, found that an average of 29.8 mg. apparent salicylate was excreted by his group one hour after ingestion of the drug. The excellent agreement between these values lends further credence to the significance of the excretion *vs.* dissolution rate curve.

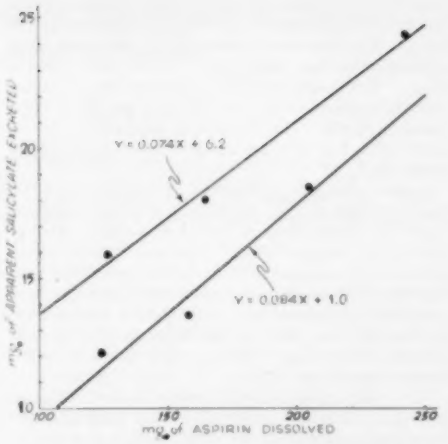

Fig. 1.—Plots of mean amount of apparent salicylate excreted (one hour after the administration of two 0.3-Gm. aspirin tablets) against *in vitro* dissolution rate (mean amount of aspirin dissolved in ten minutes from one tablet). Lower line, group *A* (fourteen subjects); upper line, group *B* (ten subjects).

Some Variables Affecting Comparative *In Vivo* Evaluation of Aspirin Absorption.—The experimental findings and some of the work of other investigators which has been previously cited make it possible to recognize several factors which could influence the result of clinical studies. One of these factors is the dissolution rate of the particular dosage form. The fact that different brands of aspirin may differ in dissolution characteristics and, therefore, in the rapidity of absorption has been largely disregarded. This may account for some of the conflicting clinical reports concerning the relative advantages of plain and "buffered" aspirin.

It is also necessary to recognize those experimental conditions which are conducive to the demonstration of product-to-product differences as well as those conditions which tend to submerge such differences. Anything that could bring about rapid gastric emptying would serve to mask product-to-product differences. Administration of the tablets on full stomach would probably have the same effect, despite the fact that food delays gastric emptying. Administration of finely crushed tablets or requesting patients to chew the tablets would also diminish differences between products. On the other hand, administration of whole tablets (in fairly large doses to reduce the effect of variations in blank readings), on empty stomach and with relatively small amounts of liquid, would accentuate the differences among products. This writer has no quarrel with investigators employing experimental procedures representative of one or the other extreme; it is only hoped that this discussion may lead to a better understanding and interpretation of their experimental results.

The Significance of the Tablet Disintegration Test.—Rapid disintegration of tablets has been equated by some workers with rapid availability of the drug for absorption. The unfortunate

practice of using the term disintegration when speaking of dissolution has further complicated the issue. The important prerequisite for rapid drug absorption is fast dissolution of the solid medicament. While delayed disintegration can interfere with absorption, it does so only by its effect upon dissolution rate. This has been pointed out previously (23), but the implications have been largely overlooked.

A good example is provided by the experimental results of this study. Comparison of disintegration times and dissolution or initial absorption rates of the various products tested indicates that the faster absorbed products actually had the longer disintegration time. In this instance, disintegration times have no relation whatsoever to biological availability. Such a reasoning does not, of course, apply to enteric coated tablets, where disintegration time can be the determining factor (19).

The disintegration test would not distinguish between rapidly and slowly dissolving small granules or primary particles. It would not, for instance, reflect any changes in dissolution rate due to a change, with time, in the crystalline character of polymorphic compounds. For example, it has been reported that amorphous novobiocin is soluble, readily absorbed, and biologically active, while the crystalline form is only slightly soluble and biologically inactive (24). Amorphous novobiocin in the dry state may slowly revert to the crystal form on standing (25). Such a transformation and inactivation, biologically speaking, would not readily change the disintegration characteristics of novobiocin tablets. However, this change would readily be recognizable by its effect on dissolution rate.[1]

While the tablet disintegration test may be useful as an industrial control procedure, it has no value as an index of the biological availability of compressed tablets. The U. S. P. makes no such claim, but the significance of the disintegration test, or rather the lack thereof, is not fully appreciated. It is suggested that the U. S. P. tablet disintegration test be replaced by a dissolution test for compressed tablets. In the choice of dissolution medium for such a test, the specific effects of pH, enzymes, volume, bile salts, etc. on a given drug must be taken into account where necessary.

Since tablet formulation studies have, so far, been concerned with the effect of formulation variables upon the disintegration rather than upon the dissolution properties of compressed tablets, there is need to study the effect of tablet formulation factors upon dissolution rate. Such studies are currently in progress in this laboratory.

[1] It should be possible to demonstrate similar dissolution and absorption rate dependence upon crystal state with riboflavin, since this drug exhibits wide variation in solubility due to differences in crystalline structure.

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Colorimetric Determination of Phenylephrine Using 4-Aminoantipyrine

By C. F. HISKEY and N. LEVIN†

A study of the coupling reaction between 4-aminoantipyrine and phenylephrine in alkaline ferricyanide solutions is reported herein. A number of important variables were studied to determine their effect on the kinetics and extent of the reaction. A procedure for the determination of phenylephrine in some pharmaceutical preparations is also included.

RECENTLY it became desirable to have a simple colorimetric determination for phenylephrine in the presence of a mixture of nitrogenous bases such as pyrilamine, dihydrocodeinone, etc., that interfered with the usual bromination method (1) and the coupling reaction with diazotized *p*-nitroaniline (2).

Since phenylephrine is a phenol with the *para*-position available for coupling with 4-aminoantipyrine, it was decided to try to apply the reaction in this instance.

The utility of this reaction for analytical purposes has been extensively investigated (3–8) since its discovery by Emerson (9, 10) in 1938. Recently Johnson and Savidge (11) reviewed its applicability to a wide range of pharmaceutical products.

EXPERIMENTAL

In our preliminary work we used the conditions outlined by Johnson and Savidge for phenols giving 4-aminoantipyrine dyes insoluble in chloroform. The first results obtained were not encouraging, since the color development was very weak and not reproducible. By changing the order of addition so that the phenylephrine, the 4-aminoantipyrine, and the ferricyanide were first combined and then diluted to volume with buffer, a set of conditions satisfactory for the assay were found.

Reagents.—4-Aminoantipyrine, mol. wt. 203.25, purchased from Eastman Organic Chemicals and used without further purification. Solutions (0.025 *M*) were prepared daily.

Potassium ferricyanide C. P., 0.010607 *M* (2 Gm./100 ml.) prepared daily.

Phenylephrine hydrochloride solution, 200 mg./100 ml. in water. Sodium bicarbonate buffer, 0.01 *M*.

Preliminary Observation.—A preliminary measurement of the absorption spectrum of the coupled phenylephrine was effected and compared with that of the reagent blanks to establish whether any interference was to be expected. The procedure employed consisted of adding 3 ml. of the phenylephrine standard to a 100-ml. volumetric flask, followed, in turn, by 1 ml. of the ferricyanide solution and then by 2 ml. of the aminoantipyrine solution. The solution was then brought to volume immediately with 2.5×10^{-3} *M* sodium carbonate. The reagent blank was prepared similarly, except that the phenylephrine was omitted.

It was observed that as the aminoantipyrine was admitted to the solution, an intense red color was formed, which in the case of the reagent blanks was discharged shortly after the addition of the carbonate solution. When the phenylephrine was present the red color diminished in intensity as the carbonate was added but did not fade completely.

In Fig. 1, the plotted absorption spectra of these two solutions, using water as a reference, show that the solution containing phenylephrine (*A*) has a well defined peak at 500 mμ, at which wavelength the reagent blank (*B*) has practically zero absorbance. The residual spectrum in the reagent blank was shown to be due to excess ferricyanide and not to any reaction by-product.

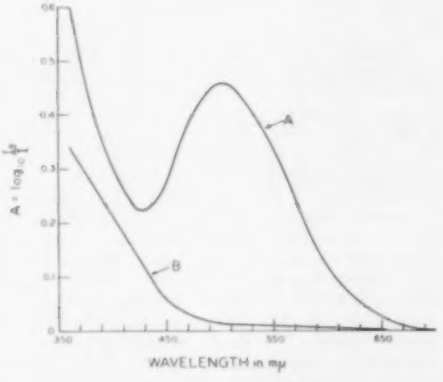

Fig. 1.—Spectra of phenylephrine (*A*) and reagent blank (*B*) under one set of reaction conditions.

Absorption Spectra of Reaction Intermediates.—The appearance of an intense red color when the reagent blank was prepared suggested further investigation of this process. Accordingly, 5 ml. of the potassium ferricyanide was mixed with the 4-aminoantipyrine reagent solution in a 100-ml. volumetric flask. As soon as the red color had formed the solution was diluted to volume with 0.01 *M* sodium bicarbonate solution. This diluted solution was then transferred to an optical cell placed in the sample side of the DK-2 spectrophotometer. As

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reference, 0.01 *M* sodium bicarbonate solution was used. The spectra were determined at various time intervals after mixing. It was observed that the red substance had an absorption maximum at 525 mμ in contrast with the 500 mμ maximum observed for the phenylephrine dye.

In Fig. 2 the absorbance at 525 mμ of the solution is plotted as a function of time. It is seen that in fifty minutes the red compound has completely decomposed.

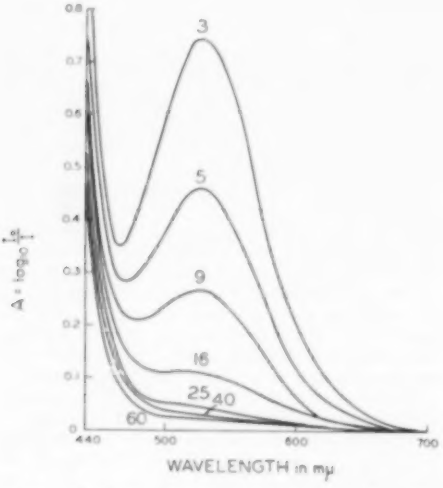

Fig. 2.—Spectrum of the reagent blank as a function of time in minutes.

A similar study was performed incorporating phenylephrine into the solution. This was done by taking 3 ml. of the phenylephrine standard and adding to it, in turn, 5 ml. of the ferricyanide and 5 ml. of the aminoantipyrine reagent. As soon as the red color had formed, the solutions were diluted to volume with 0.01 *M* sodium bicarbonate. Again the spectra were measured in the DK-2 using 0.01 *M* sodium bicarbonate in the reference beam. In Fig. 3, we see that the red color has an absorption peak close to, but not exactly at, 525 mμ. With time, the fading of this absorption peak is quite rapid and it will be noticed that there is a gradual shift away from the 525 mμ region to the 500 mμ region. After this had been accomplished the rate of fading with time diminished appreciably and there was no further spectral shift associated with that fading.

These data are somewhat better understood if one plots the peak absorption in the 500–525 mμ region for both of these experiments as a function of time. When this is done, the rapid decline to background when phenylephrine is absent is noteworthy. When phenylephrine is present the decrease in absorbance is exceedingly rapid for the first fifteen minutes, after which the slope of the curve changes abruptly to a much lower rate.

It appears that in the absence of phenylephrine the 4-aminoantipyrine reacts to form a red intermediate complex or free radical which decomposed to a colorless substance in a relatively short period of

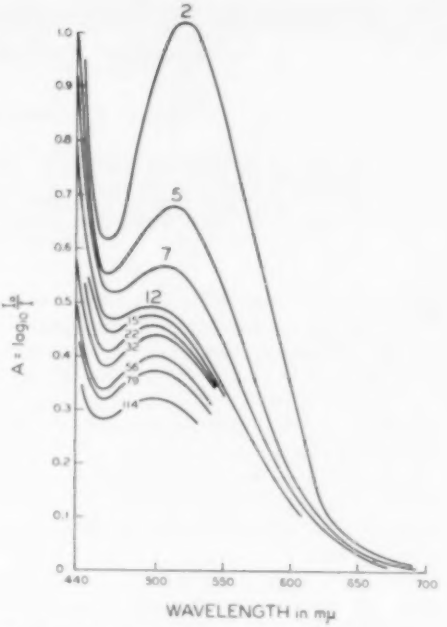

Fig. 3.—Spectrum of reagent and phenylephrine as a function of time.

time. However, when phenylephrine is present this intermediate is able to interact with the phenylephrine to form a much more stable reaction product. The antipyrine phenylephrine moiety, while not completely stable under the conditions of this reaction, nevertheless has a rate of decomposition sufficiently slow to enable us to make a practical use of it in this method of assay. If a period of time of about fifteen to thirty minutes is allowed to elapse before the readings are taken, the red intermediate of aminoantipyrine will have completely disappeared, leaving a red color due only to the coupled phenylephrine.

The Role of Concentration Ratios.—A study was made next of the effect of the ferricyanide and the 4-aminoantipyrine concentrations on the absorbance. In general these data were taken by combining the indicated amounts of the reagent solutions in a 100-ml. flask, after which the solution was diluted to volume with 0.01 *M* bicarbonate. The solutions were allowed to stand for fifteen minutes, after which the absorbance of the solution was measured at 500 mμ on a Beckman DU spectrophotometer using the 0.01 *M* bicarbonate solution as a reference standard. The data so obtained are presented in Table I.

These data reveal that both the ferricyanide and the aminoantipyrine concentrations are important variables affecting the color intensity. From the data, where 0.5 and 1 ml. of ferricyanide were used with three concentrations levels of aminoantipyrine, it appears that with too great a ratio of aminoantipyrine to ferricyanide, most of the oxidant is used up in forming the unstable red complex of the aminoantipyrine and little is available for the coupling

TABLE I.—EFFECT OF REAGENT CONCENTRATION[a] ON ABSORBANCE

4-Amino-antipyrine Soln., ml.	Ferricyanide Soln., ml. →	0.5	1.0	1.5	2.0	3.0	5.0
1.0		0.350	0.532	0.533	0.472	0.421	...
2.0		0.283	0.512	0.670	0.725	0.699	0.675
3.0		0.175	0.430	0.570	0.695	0.771	0.780
5.0		...	...	...	0.604	0.758	0.875

[a] Phenylephrine concentration 1.20 mg./100 ml. (5.9×10^{-5} *M*).

TABLE II.—TABULATION OF DATA

Phenylephrine mg./100 ml.	0.1 *M* HCO_3^-		0.01 *M* HCO_3^-		Hycomine Syrup	
	A	*A*(1 %, 1 cm.)	*A*	*A*(1 %, 1 cm.)	*A*	*A*(1 %, 1 cm.)
0.03	...	..	0.014	466	...	..
0.05	0.022	440	...	..	...	..
0.06	...	..	...	..	0.037	616
0.15	...	..	0.100	666	...	..
0.20	0.077	385	...	..	0.139	695
0.30	...	..	0.206	687	...	..
0.40	0.154	385	...	..	0.276	690
0.60	0.238	397	0.408	680	0.411	685
0.80	0.301	376	...	..	0.540	675
0.90	...	..	0.602	669	...	..
1.0	0.392	392	...	..	...	..
1.20	0.468	389	0.797	664	0.779	650

reaction. Hence, with increasing amounts of aminoantipyrine, less color is produced by the phenylephrine.

If the concentration of aminoantipyrine is held constant at 2 ml. while the ferricyanide is increased, the degree of coupling is increased, reaching a maximum when equal number of milliliters of reagents are added, corresponding to a ratio of about 2.5 moles of ferricyanide to one of aminoantipyrine. In general, when this ratio is obtained at any other concentration level a maximum amount of coupled phenylephrine is achieved. This is illustrated by the values when 1:1, 2:2, and 3:3 ml. are taken of the two reagents. As the reagents are increased maintaining the ratio of 2.5, leveling off of intensity occurs when the 5:5-ml. level is approached. This appears to be a practical working concentration with the reagent blank having an appreciable absorbance, i.e., 0.06–0.07, and with *A* (1 cm., 1%) for the phenylephrine being about 700. Consequently, these were the conditions that were finally adopted for this assay.

Test of the Procedure.—A test of the procedure was made by measuring the conformity of the reaction to the Bouger-Beer absorption law for both standard solutions and a pharmaceutical preparation. The pharmaceutical was a sugar-sorbo base syrup containing in addition to phenylephrine, the lower esters of *p*-hydroxy benzoic acid, pyrilamine maleate, dihydrocodeinone bitartrate, and homatropine methyl bromide.

The following procedure was employed: Transfer a sample containing 0.03 to 1.20 mg. of phenylephrine to a 100-ml. volumetric flask, add 5 ml. of ferricyanide solution followed by 5 ml. of 4-aminoantipyrine solution. Dilute the solutions to mark with 0.01 *M* bicarbonate buffer and allow to stand for fifteen minutes, then read the absorbance at 500 mμ against a reagent blank.

In Table II, some of the data are tabulated, including a set of results obtained when 0.1 *M* bicarbonate was substituted for the 0.01 *M* buffer.

In all three sets of data it will be observed that agreement with the absorption law is within a few per cent when the absorbance is 0.2 or greater. The pronounced effect of the buffer concentration is apparent from a comparison of the first two sets of data. At the higher concentration of buffer the sensitivity of the reaction is only about 60% of that for the lower concentration.

The precision and reproducibility in all cases is adequate for control operations. In addition, the phenylephrine determination can be made directly upon the sample without any separative steps. This contributes substantially to the simplicity of this procedure.

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Veratrum Alkaloids XLVI

Structure-Activity Relationships in a Series of Analogs of the Protoveratrines

By S. MORRIS KUPCHAN, JOHN C. GRIVAS, C. IAN AYRES, LALIT J. PANDYA, and LAWRENCE C. WEAVER†

In view of the significant pharmacological differences between protoveratrine A and protoveratrine B when the substances are administered orally, and of our recent revelation that the structures of the two compounds differ only in the nature of the acid moiety of the ester at C_3, a series of analogs of the protoveratrines have been prepared and subjected to preliminary pharmacological evaluation. The results indicate that considerable alterations can be made in the structure of the ester affixed at C_3 without greatly altering hypotensive potency.

PROTOVERATRINE A and protoveratrine B, two ester alkaloids isolated from a number of veratrum species, have been shown to be useful agents in the treatment of hypertension (1, 2). The limiting factor in the clinical use of these drugs has been the narrow dosage range between hypotensive and emetic effects.

Recent clinical studies have established significant differences between protoveratrine A and protoveratrine B when the substances are administered orally (3–5). Protoveratrine A is a potent hypotensive agent with a narrow therapeutic dosage range. Protoveratrine B, on the other hand, is inactive orally in doses several times the hypotensive doses of protoveratrine A. However, studies of divided doses of more than 10 mg. a day have indicated that protoveratrine B has strong hypotensive activity, which may be prolonged and not accompanied by emetic effects.

Structural studies in our laboratory recently culminated with the elucidation of the complete structures and configurations of protoveratrine A (I) and protoveratrine B (II) (6). It is evident that the structures of the two compounds are exceedingly similar and, indeed, differ only in the nature of the acid moiety of the ester at C_3. In view of the aforementioned pronounced difference in activity between protoveratrine A and protoveratrine B, it was deemed desirable that additional compounds which differ from the protoveratrines solely in the nature of the acid residue affixed at C_3 should be prepared and subjected to pharmacological evaluation. The present report details the synthesis and preliminary pharmacological evaluation of twenty-five analogs of the protoveratrines.

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DISCUSSION

Protoveratrine B was the starting material for the preparation of all the new compounds reported herein. In one approach, the first step consisted of selective cleavage of the 2′,3′-dihydroxy-2′-methylbutyrate residue from C_3 by sodium periodate oxidation (6). The triester derivative which resulted, protoverine 6,7-diacetate 15-(*l*)-2′-methylbutyrate, proved to be an invaluable intermediate for elaboration of interesting compounds. For convenience, the latter compound has been assigned the name "desatrine," derived from *des*acylprotover*atrine*. Our earlier studies of selective acylation of related compounds had shown that the hydroxyl group at C_3 is more reactive than the C_{15}-hydroxyl (7). Acylation of desatrine under controlled conditions afforded a series of 3,6,7,15-tetraester analogs of the protoveratrines, Compounds IV–XV. Amination of desatrine 3-chloro-acetate (X) with diethylamine yielded desatrine N,N-diethylaminoacetate (XVI). Direct partial acylation of protoveratrine B yielded another series of protoveratrine analogs (XVII–XXIV). Our earlier work had revealed that controlled acylation of protoveratrine B led to selective reaction at the secondary hydroxyl group of the 2′,3′-dihydroxy-2′-methylbutyrate residue affixed at C_3 (6).

As indicated in Fig. 1, treatment of desatrine 3-(3′-tosyloxy-2′-hydroxy-2′-methylbutyrate (XXIV) (6) with methanol at reflux temperature or with potassium iodide in acetonitrile for five hours afforded a sulfur-free product. The latter product was assigned the desatrine 3-(2′,3′-epoxy-2′-methylbutyrate) structure (XXV) on the basis of its empirical formula and reactivity. Upon treatment with *p*-toluenesulfonic acid in acetonitrile, XXV reverted to XXIV. Reaction of XXV with anhydrous hydrogen chloride yielded a hydrogen chloride adduct. The latter compound consumed 1 oxygen equivalent of chromic acid, in accord with the desatrine 3-(3′-chloro-2′-hydroxy-2′-methyl-

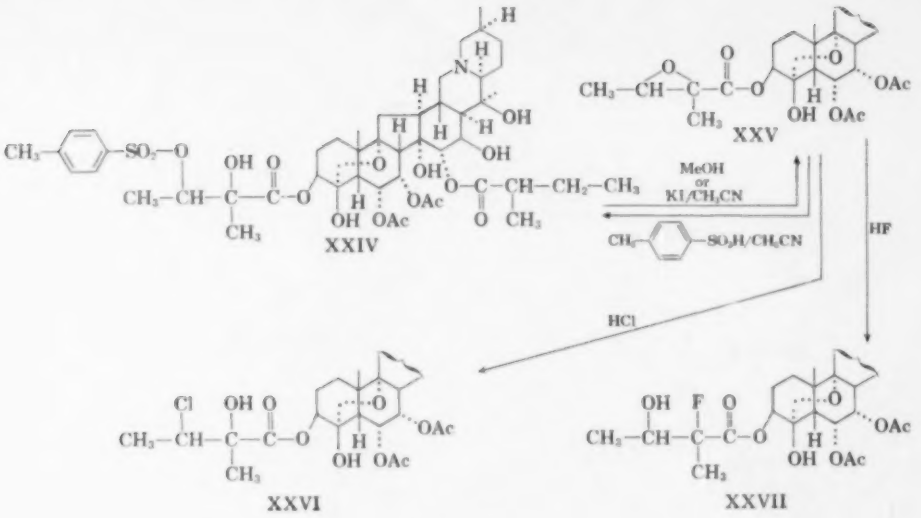


Figure 1

butyrate) structure (XXVI). Reaction of XXV with anhydrous hydrogen fluoride yielded a product assigned the desatrine 3-(3′-hydroxy-2′-fluoro-2′-methylbutyrate) structure (XXVII), in agreement with the observed chromic acid consumption of 2 oxygen equivalents. Amination of XXIII with diethylamine yielded desatrine 3-(3′-N,N-diethylaminoacetoxy-2′-hydroxy-2′-methylbutyrate) (XXVIII) and catalytic reduction of XXI gave desatrine 3 - (3′ - (4″ - aminobenzoxy) - 2′ - hydroxy - 2′ - methylbutyrate) (XXIX).

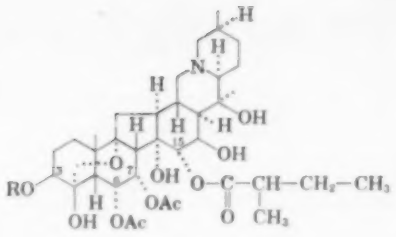
Twenty-six new compounds (see Table I and Fig. 2) have been investigated for hypotensive activity and the results are listed in Table II along with comparable earlier results for protoveratrine A (I), protoveratrine B (II), and escholerine (III) (8, 9).[1] The methods used have been described (10, 11). Adult mongrel dogs, unselected as to sex, were employed in all experiments described. Anesthesia was maintained at upper plane ii or lower plane i of Stage III by the judicious use of sodium pentobarbital. Test drug solutions were freshly prepared and injected intravenously. The ability of these drugs to lower systemic blood pressure and, in most cases, to decrease the carotid occlusion response have been investigated; the carotid occlusion response is an added indication of activity for this type of compound.

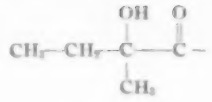
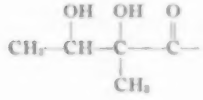
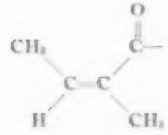
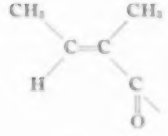
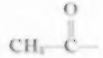
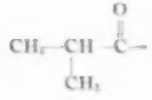
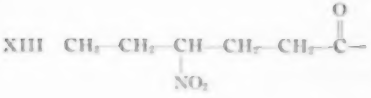
In our previous studies the compounds examined were protoverine derivatives with varying substitution at positions 3, 4, 6, 7, 14, 15, and 16 (7, 12). The results supported the following generalizations: (*a*) esterification at position 16 with acetate or isobutyrate is accompanied by a profound loss in activity, (*b*) esterification at positions 3 and 15 is required for high activity, (*c*) esterification at position 15 with a branched-chain acid is advantageous, (*d*) the ester grouping at position 3 need not be branched, (*e*) positions 6 and 7 need not be esterified for good activity, (*f*) esterification at position 7 with a branched-chain acid may be disadvantageous, (*g*) oxidation of the alcohol group at position 16 to a ketone group is accompanied by a loss in activity, (*h*) acetonide formation at positions 14 and 15 is accompanied by a profound loss in activity, (*i*) esterification at position 4 may be disadvantageous.

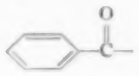
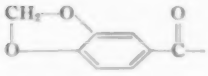
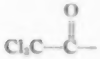
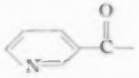
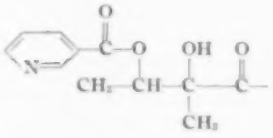
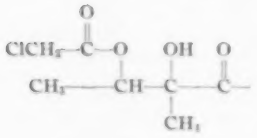
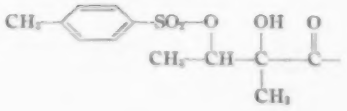
The protoveratrine analogs listed in Table II all have structures which generally fit the aforementioned "requirements" for high hypotensive activity, and, in accord with expectations, the compounds showed a high average order of activity. Among the series prepared by acylation of desatrine (i.e., compounds IV–XV), only the 3-diethylphosphate (XV) had suffered a profound loss of activity. The 3-acetate (V), 3-angelate (III), 3-trichloroacetate (IX), 3-tosylate (XIV), and 3,4′-nitrobenzoate (XII) show slightly diminished activity relative to the protoveratrines. There is not sufficient data to differentiate quantitatively among the remaining compounds produced by acylation of desatrine. Among the compounds derived directly from protoveratrine B (i. e., compounds XVII–XXIV), some decrease in activity was observed in the 3′-acetoxy-2′-hydroxy-2′-methylbutyrate (XVII) and the 3′-(4″-nitrobenzoxy) - 2′ - hydroxy - 2′ - methylbutyrate (XXI). The remaining compounds were not sufficiently different from protoveratrine B to indicate significant alterations in activity.

It is apparent from the data reported herein that considerable alterations can be made in the structure of the ester moiety affixed at position 3 in analogs of protoveratrine without greatly altering hypotensive potency. A future report will present data on the emetic properties of these compounds as they relate to the hypotensive activity in unanesthetized dogs.

[1] We are indebted to Shirley Study and Olive Johnston for technical assistance in pharmacology.



Compound	R
I	
II	
III	
IV	
V	
VI	
XIII	
XIV	
XV	

Compound	R
VII	
VIII	
IX	
X	
XI	
XII	
XXII	
XXIII	
XXIV	

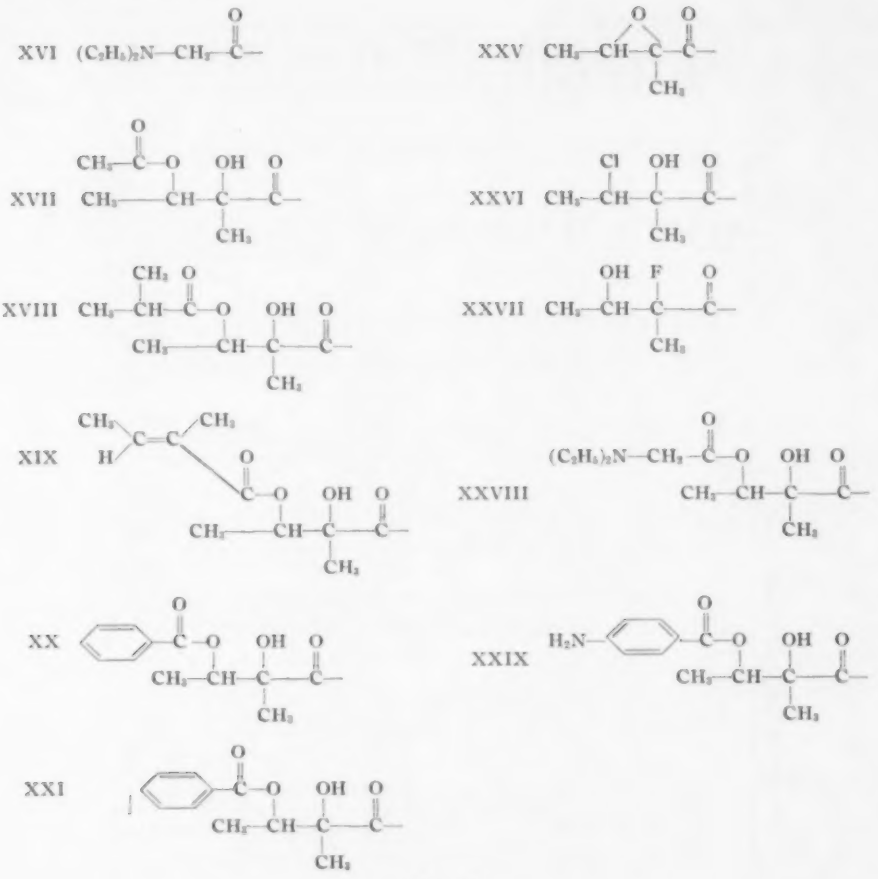


Figure 2.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Infrared spectra were determined in chloroform on a Baird double beam recording spectrophotometer. Microanalyses[3] were carried out on samples dried under reduced pressure at 110°. "Petroleum ether" refers to the fraction of boiling point 60–80°.

Acylations.—The general procedure for the acylation reactions involved portionwise addition of acyl halide (2–3 equivalents) to a stirred solution of the dry alkaloid alcohol in 10 parts of dry reagent grade pyridine at ice bath temperature. The flask was protected from moisture with a calcium chloride tube and was allowed to warm gradually to room temperature. The course of the reaction was followed by paper chromatography[4] and interrupted when suitable, usually after twenty to forty-eight hours. The solution was treated with chloroform, ice water, and dilute ammonium hydroxide to pH 8–9. The solution was extracted with chloroform four times; the combined chloroform extracts were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. To remove all traces of pyridine, the residue was repeatedly dissolved in benzene and evaporated to dryness. Occasionally, the paper chromatographic behavior of the crude residue was sufficiently promising so that direct crystallization at this point was attempted. More often, the mixtures obtained were separated by chromatography on Merck acid-washed alumina (20–25 Gm. per Gm. of alkaloid mixture). The solvent mixtures were selected on

[3] Microanalyses were made by Dr. S. M. Nagy and his associates at Massachusetts Institute of Technology.

[4] The solvent systems used were those of Levine, J., and Fischbach, H., THIS JOURNAL, 44, 543(1955): (a) *n*-butyl acetate:*n*-butanol:formic acid (25:5:1 by volume); (b) the solution prepared by adding 1 cc. of formic acid to the separated solvent layer of the system *n*-butyl acetate:*n*-butanol:water (10:25:10 cc.).

TABLE I.—CHARACTERISTICS OF NEW COMPOUNDS

No.	Desatrine Derivative (3-Substituent)	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	M. p., °C. (decompn.)	$[\alpha]_D^{25}$ pyr.	Yield, %	Crystal Form
IV	Tiglate[c]	$C_{31}H_{51}NO_{13}$	62.76[a]	62.87	7.96[a]	7.75	219–220	−23	24	ellipsoids
V	Acetate[c]	$C_{28}H_{47}NO_{13}$	60.53[b]	60.67	7.88[b]	7.71	253–254	−35	15	needles
VI	Isobutyrate[c]	$C_{30}H_{51}NO_{13}$	62.88	62.81	8.04	8.26	188–190	−42	16	prisms
VII	Benzoate	$C_{33}H_{49}NO_{13}$	64.00[a]	64.21	7.49[a]	7.14	240–241	−4	42	prisms
VIII	3′,4′-Methylene dioxybenzoate	$C_{34}H_{49}NO_{15}$	62.76	62.91	7.06	7.13	221–222	+23	50	micro needles
IX	Trichloroacetate[d]	$C_{30}H_{44}Cl_3NO_{13}$	54.57	54.67	6.50	6.31	185–190	−40	20	prisms
X	Chloroacetate[e]	$C_{30}H_{46}ClNO_{13}$	59.25	59.77	7.33	7.45	190–205	−39	22	prisms
XI	Nicotinate[f]	$C_{32}H_{48}N_2O_{13}$	62.42[a]	62.66	7.36[a]	7.26	237–239	−5	51	prisms
XII	4′-Nitrobenzoate[g]	$C_{33}H_{48}N_2O_{15}$	61.27	61.11	6.94	7.06	175–177	+11	45	needles
XIII	4′-Nitrohexanoate[h]	$C_{32}H_{54}N_2O_{15}$	60.27	60.11	7.71	7.58	187–188	−34	38	prisms
XIV	Tosylate[i]	$C_{33}H_{51}NO_{15}S$	60.90	60.86	7.25	7.36	213–215	−15	18	prisms
XV	Diethylphosphate[j]	$C_{32}H_{54}NO_{15}P$	57.88	58.23	7.77	7.77	203–204	−31	23	needles
XVI	N,N-Diethylaminoacetate[k]	$C_{32}H_{56}N_2O_{12}$	62.51	63.22	8.24	8.12	189–190	−40	43	rosettes
XVII	3′-Acetoxy-2′-hydroxy-2′-methylbutyrate[c]	$C_{33}H_{51}NO_{16}$	60.61	60.27	7.70	7.99	230–231	−18	29	needles
XVIII	3′-Isobutyroxy-2′-hydroxy-2′-methylbutyrate	$C_{35}H_{59}NO_{16}$	61.42	61.76	7.84	7.95	217–218	−21	61	needles
XIX	3′-Tigloxy-2′-hydroxy-2′-methylbutyrate[l]	$C_{37}H_{57}NO_{16}$	62.30	62.02	7.90	7.76	176–178	−19	32	rosettes
XX	3′-Benzoxy-2′-hydroxy-2′-methylbutyrate	$C_{38}H_{57}NO_{16}$	63.02	63.16	7.32	7.30	177–179	−22	71	plates
XXI	3′-(4″-Nitrobenzoxy)-2′-hydroxy-2′-methylbutyrate[m]	$C_{38}H_{56}N_2O_{18}$	59.02[b]	59.10	6.97[b]	6.65	169–171	−14	56	plates
XXII	3′-Nicotinoxy-2′-hydroxy-2′-methylbutyrate[n]	$C_{37}H_{56}N_2O_{16}$	60.52[b]	60.20	7.30[b]	7.01	162–163	−18	32	plates
XXIII	3′-Chloroacetoxy-2′-hydroxy-2′-methylbutyrate[o]	$C_{35}H_{54}ClNO_{16}$	58.56	59.43	7.42	7.21	201–203	−18	55	needles
XXV	2′,3′-Epoxy-2′-methylbutyrate[p]	$C_{31}H_{51}NO_{14}$	62.18	62.47	7.77	7.78	227–228	−26	40	prisms
XXVI	3′-Chloro-2′-hydroxy-2′-methylbutyrate[q]	$C_{31}H_{52}ClNO_{14}$	59.44	59.21	7.54	7.37	262–263	−37	53	prisms
XXVII	3′-Hydroxy-2′-fluoro-2′-methylbutyrate	$C_{31}H_{52}FNO_{14}$	60.65	60.87	7.70	7.78	257–259	−41	34	prisms
XXVIII	3′-N,N-Diethylaminoacetoxy-2′-hydroxy-2′-methylbutyrate[r]	$C_{37}H_{74}N_2O_{16}$	61.17	61.24	8.03	7.98	203–205	−19	60	plates
XXIX	3′-(4″-Aminobenzoxy)-2′-hydroxy-2′-methylbutyrate[s]	$C_{38}H_{58}N_2O_{16}$	61.48[a]	61.29	7.36[a]	7.43	193–195	−28	73	plates

[a] Calcd. with ½ H_2O. [b] Calcd. with 1 H_2O. [c] Equivalents of volatile acid found: 3.7. [d] Chlorine: calcd. 12.72; found, 12.79. [e] Chlorine: calcd., 4.60; found, 4.67. [f] Nitrogen: Calcd., 3.46; found, 3.60. [g] Nitrogen: calcd., 3.33; found, 3.08. [h] Nitrogen: calcd., 3.35; found, 3.08. [i] Sulfur: calcd., 3.78; found, 3.74. [j] Phosphorus: calcd., 3.73; found, 3.42. [k] Equivalents of volatile acid found: 2.9; nitrogen: calcd., 3.47; found, 3.60. [l] Prepared by Dr. A. D. J. Balon. [m] Nitrogen: calcd., 2.92; found, 2.98. [n] Nitrogen: calcd., 3.00; found, 3.10. [o] Calcd. with 1 CH_3COCH_3; chlorine: calcd., 3.76; found, 4.28. [p] Equivalents of volatile acid found: 2.7. [q] Chlorine: calcd., 4.28; found, 4.74. [r] Nitrogen: calcd., 3.04; found, 2.81. [s] Nitrogen: calcd., 3.02; found, 3.53.

TABLE II —COMPARATIVE HYPOTENSIVE ACTIVITY OF ANALOGS OF THE PROTOVERATRINES IN ANESTHETIZED DOGS

Compound No.	Desatrine Deriv. (3-Substituent)	No. Dogs	Dose γ/Kg.	Blood Pressure Chg., %	Blood Pressure Dur., min.	Carotid Occlusion Chg., %	Carotid Occlusion Dur., min.
I	2′-Hydroxy-2′-methylbutyrate	2	2	−49	>83	−68	>90
II	2′,3′-Dihydroxy-2′-methylbutyrate	2	2	−46	26	−55	38
III	Angelate	2	32	−47	>150	−90	>158
IV	Tiglate	2	2	−37	23	−50	30
V	Acetate	3	32	−44	>103	−80	>104
VI	Isobutyrate	3	8	−48	>130	−93	>94
VII	Benzoate	3	8	−40	>60	−100	>120
VIII	3′,4′-Methylenedioxybenzoate	2	8	−51	167	−100	230
IX	Trichloroacetate	4	32	−64	33	−38	80
X	Chloroacetate	1	2	−67	>120	−50	30
		2	4	−60	>76	0	...
XI	Nicotinate	2	2	−29	25	−36	38
XII	4′-Nitrobenzoate	4	8	−22	10	−15	60
XIII	4′-Nitrohexanoate	2	4	−64	>218	−82	142
XIV	Tosylate	1	8	−40	28	−6	32
		1	16	−55	210	−14	37
XV	Diethylphosphate	1	32	−7	4	0	...
		1	128	−69	16	0	...
XVI	N,N-Diethylaminoacetate	1	4	−19	33	−25	16
		1	8	−58	18	−100	21
XVII	3′-Acetoxy-2′-hydroxy-2′-methylbutyrate	2	16	−28	>66	−4	63
XVIII	3′-Isobutyroxy-2′-hydroxy-2′-methylbutyrate	3	4	−40	>70	−85	>105
XIX	3′-Tigloxy-2′-hydroxy-2′-methylbutyrate	2	8	−20	255	−57	138
XX	3′-Benzoxy-2′-hydroxy-2′-methylbutyrate	3	4	−25	>120	−90	187
XXI	3′-(4″-Nitrobenzoxy)-2′-hydroxy-2′-methylbutyrate	1	8	−20	84	−58	54
		2	16	−35	>108	−50	40
XXII	3′-Nicotinoxy-2′-hydroxy-2′-methylbutyrate	2	4	−20	130	−100	25
XXIII	3′-Chloroacetoxy-2′-hydroxy-2′-methylbutyrate	2	8	−45	53	−96	41
XXIV	3′-Tosyloxy-2′-hydroxy-2′-methylbutyrate	1	8	−23	>160	−57	>180
XXV	2′,3′-Epoxy-2′-methylbutyrate	1	16	−37	>120	−92	>96
		1	32	−79	>166	−100	>166
XXVI	3′-Chloro-2′-hydroxy-2′-methylbutyrate	2	2	−48	35	−69	65
XXVII	3′-Hydroxy-2′-fluoro-2′-methylbutyrate	2	4	−66	>134	−67	55
XXVIII	3′-N,N-Diethylaminoacetoxy-2′-hydroxy-2′-methylbutyrate	2	4	−24	80	−90	60
XXIX	3′-(4″-Aminobenzoxy)-2′-hydroxy-2′-methylbutyrate	2	8	−28	>113	−100	60

the basis of the R_f values of the alkaloids, and generally ranged from benzene, benzene-chloroform, and chloroform, to mixtures of chloroform-methanol containing gradually increasing proportions of methanol. The acylations were usually accompanied by some discoloration. The colored material was generally small in quantity and the major proportion was either retained by the alumina or eluted with the first few fractions. Initial fractions were usually kept very small in order to separate the yellow or brown impurities in the forerun from easily eluted colorless alkaloids. Most of the compounds were crystallized from acetone-petroleum ether. The only exceptions were VI (petroleum ether), VII (ether-petroleum ether), and XXIX (benzene-petroleum ether). Products were recrystallized for analysis at least once from the same solvents.

For the acylations involving chloroacetyl chloride (i. e., for the preparation of compounds X and XXIII), benzene was used as solvent. Extensive decomposition and color formation was observed in trial runs in pyridine.

Desatrine 3-(N,N-Diethylaminoacetate) (XVI).—A solution of desatrine 3-chloroacetate (260 mg.) (X) in dry benzene (10 cc.) was treated with diethylamine (1 cc.) and the mixture was allowed to stand in a stoppered flask at room temperature for forty-eight hours. Evaporation to dryness under reduced pressure yielded a residue which was treated with dilute ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The amorphous residue was crystallized from acetone-petroleum ether to yield colorless rosettes. The crystalline product was shown by paper chromatography to be homogeneous.

Desatrine 3-(2′,3′-Epoxy-2′-methylbutyrate) (XXV).—*By Methanolysis of Desatrine 3-(3′-Tosyloxy-2′-hydroxy-2′-methylbutyrate)* **(XXIV).**—A solution of desatrine 3-(3′-tosyloxy-2′-hydroxy-2′-methylbutyrate (XXIV) (6) (1.35 Gm.) in methanol (125 cc.) was heated under reflux for twenty-four hours. The methanol was evaporated under reduced pressure and the residue was chromatographed on Merck acid-washed alumina (30 Gm.). The column yielded to benzene-chloroform (50:50) and to chloroform a resin which was shown to be homogeneous by paper chromatography. Crystallization from ether afforded needles (550 mg.), m. p. 231–232° (decompn.). Recrystallization from acetone-petroleum ether gave prisms, m. p. 227–228° (decompn.).

By Sodium Iodide-Acetonitrile Treatment of Desatrine 3-(3′-Tosyloxy-2′-hydroxy-2′-methylbutyrate) (XXIV).—A solution of desatrine 3-(3′-

tosyloxy-2′-hydroxy-2′-methylbutyrate) (XXIV) (550 mg.) and sodium iodide (400 mg.) in acetonitrile (15 cc.) was heated under reflux for five hours. The precipitate (sodium tosylate) was filtered and the filtrate was reduced to a small volume. Water (10 cc.) was added and the solution was extracted with chloroform. The chloroform extract was dried and evaporated under reduced pressure to a resin. The resin was crystallized from acetone-petroleum ether to yield desatrine 3-(2′,3′-epoxy-2′-methylbutyrate) hydriodide in the form of needles (250 mg.), m. p. 232–233° (decompn.).

Anal.—Calcd. for $C_{31}H_{46}INO_{14}\cdot H_2O$: C, 52.53; H, 6.90; I, 13.55. Found: C, 52.42; H, 7.04; I, 13.23.

Treatment of the hydriodide with dilute ammonium hydroxide and chloroform and further workup in the usual manner yielded XXV in the form of free base. In a subsequent preparation, the hydriodide was not isolated as such; the concentrated reaction mixture was treated with chloroform-dilute ammonium hydroxide. Evaporation of the chloroform extract yielded a resin which was chromatographed on Merck acid-washed alumina. In this preparation, 710 mg. of crystalline XXV free base was obtained from 1.8 Gm. of XXIV.

Desatrine 3-(3′-Tosyloxy-2′-hydroxy-2′-methylbutyrate) (XXIV) from the Treatment of Desatrine 3-(2′,3′-Epoxy-2′-methylbutyrate) (XXV) with *p*-Toluenesulfonic Acid.—A solution of desatrine 3-(2′,3′-epoxy-2′-methylbutyrate) (XXV) (100 mg.) and *p*-toluenesulfonic acid hydrate (100 mg.) in dry acetonitrile (5 cc.) was allowed to stand at room temperature overnight. The mixture was cooled to 0°, basified to pH 8–9 with dilute ammonium hydroxide, and extracted with chloroform. The chloroform extract was dried over anhydrous magnesium sulfate and evaporated under reduced pressure to yield an amorphous residue. Crystallization from acetone-petroleum ether yielded XXIV in the form of prisms (50 mg.). The identity of the material with that obtained by tosylation of protoveratrine B was confirmed by m. p., mixed m. p., infrared spectrum, optical rotation, and paper chromatographic behavior.

Desatrine 3-(3′-Chloro-2′-hydroxy-2′-methylbutyrate) (XXVI).—A solution of desatrine 3-(2′,3′-epoxy-2′-methylbutyrate) (XXV) (200 mg.) in dry ethereal hydrogen chloride (30 cc.) and benzene (15 cc.) was allowed to stand at room temperature for eighteen hours. The solvent was evaporated to dryness under reduced pressure and the residue was treated with dilute ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous magnesium sulfate and concentrated to a small volume. Addition of petroleum ether effected crystallization. Filtration of the material followed by recrystallization from acetone-petroleum ether yielded colorless prisms (110 mg.), shown to be homogeneous by paper chromatography.

Desatrine 3-(3′-Hydroxy-2′-fluoro-2′-methylbutyrate) (XXVII).—A solution of desatrine 3-(2′,3′-epoxy-2′-methylbutyrate) (XXV) (200 mg.) in dry ether (500 cc.) containing 50% hydrofluoric acid (0.2 cc.) was allowed to stand at room temperature for three hours. Chloroform was added and the solution was concentrated to a small volume. With cooling in an ice water bath, water (5 cc.) and dilute sodium carbonate solution were added (to pH 8–9), and the mixture was extracted with chloroform. The chloroform extract was dried over anhydrous magnesium sulfate and evaporated to yield an amorphous residue (200 mg.). The residue was crystallized from acetone to yield prisms (70 mg.) shown to be homogeneous by paper chromatography.

Desatrine 3-(3′-N,N-Diethylaminoacetoxy-2′-hydroxy-2′-methylbutyrate) (XXVIII).—To a stirred solution of desatrine 3-(3′-chloroacetoxy-2′-hydroxy-2′-methylbutyrate) (XXIII) (450 mg.) in dry benzene (10 cc.), diethylamine (1.05 cc., 20 mole-equivalents) was added, and the mixture was stirred at room temperature for forty-eight hours. Workup as described above for XVI yielded an amorphous crude product (410 mg.). Chromatography on Merck acid-washed alumina (10 Gm.) yielded paper chromatographically pure product. Crystallization from acetone-petroleum ether gave yellow-white plates, m. p. 203–205° (decompn.).

Desatrine 3-(3′-(4″-Aminobenzoxy)-2′-hydroxy-2′-methylbutyrate) (XXIX).—Desatrine 3-(3′-(4″-nitrobenzoxy)-2′-hydroxy-2′-methylbutyrate) (XXI) (500 mg.) in 95% ethanol (5 cc.) was hydrogenated over platinum oxide (100 mg.) at room temperature and atmospheric pressure. In two hours two mole-equivalents of hydrogen was absorbed and hydrogen uptake ceased. The reaction mixture was filtered and the residue washed with ethanol. Evaporation of the solution under reduced pressure afforded a residue which was crystallized from benzene-petroleum ether in the form of plates, m. p. 193–195° (decompn.).

Chromic Acid Titrations.—The method used in Part XLI (13) was used. The results obtained are summarized in Table III.

Table III.—Chromic Acid Titrations

Alkaloid	Oxygen Equivalents Consumed		
	Theoretical	Found, 45 min.	Found, 90 min.
Protoveratrine A (I)	1	1.00	1.06
Protoveratrine B (II)	2	2.19	2.20
Desatrine 3-(3′-tosyloxy-2′-hydroxy-2′-methylbutyrate) (XXIV)	1	1.04	1.07
Desatrine 3-(3′-chloro-2′-hydroxy-2′-methylbutyrate) (XXVI)	1	1.02	1.05
Desatrine 3-(3′-hydroxy-2′-fluoro-2′-methylbutyrate) (XXVII)	2	2.03	2.24

Diosphenol Formation as Confirmation of Structure.—The analytical procedure of chromic acid titration followed by alkaline treatment as a criterion for protoveratrine-like structure, previously described for escholerine (14), was used to check the structures of several of the products. All of the materials tested (V, VI, X, XI, XIV, XVII, XXV) gave crude products with the characteristic diosphenol absorption spectrum, λmax. 328 mμ (ε 12,700) and λmax. 0.1 *N* NaOH 381 mμ (ε 8,800).

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Investigation of Drug Release from Solids IV

Influence of Adsorption on the Dissolution Rate

By DALE E. WURSTER and GERALD P. POLLI†

The influence of an adsorbent on the dissolution rate of a slightly soluble acidic solid was investigated. Experimental data indicated that the adsorbent was capable of increasing the dissolution rate observed in water under conditions of a decreased concentration gradient (Nernst-Brünner film theory) to the maximum rate obtained when a constant concentration gradient was maintained. The approximate amount of adsorbent required to increase the slower dissolution rate to the maximum was calculated with the aid of adsorption isotherms.

IN AN EXTREMELY large number of pharmaceutical preparations including tablets, capsules, and suspensions the drugs are found in the solid state. In addition to the active ingredients these preparations also contain substances which may or may not influence the release of the drugs from the physical system. Since many of the inactive ingredients are capable of adsorbing drugs and since dissolution of the drugs takes place following their introduction into the body, it is of interest to know the effect of adsorption on the dissolution rate. The contents of the gastrointestinal tract as well as stomach and intestinal walls may also be considered as potential adsorption sites for the drug molecules. The adsorption of many drugs of varying chemical structure by various adsorbents has been reported by several workers (1–8).

An attempt was made in this investigation to determine the influence of an adsorbent on the dissolution rate of a slightly soluble acidic solid.

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PLAN OF STUDY

It was assumed in this study that dissolution of the solid solute involved a diffusion controlled process based on the Nernst-Brünner film theory (9–11). As the dissolution process takes place the concentration of the solute in solution increases, the concentration gradient, therefore, decreases, and subsequently the solution rate also decreases. If an adsorbent was present in the solution, solute molecules would be adsorbed from solution onto its surface. If, due to adsorption, the solute molecules were removed from the bulk solution, the concentration gradient would not decrease and thus, the dissolution rate, theoretically, would also not decrease. Therefore, if sufficient adsorbent is added to the bulk solution, the solution rate of a solid in its own solution should be equal to the solution rate when the concentration gradient is constant. Using adsorption isotherms, the amount of adsorbent required to produce this increase in the dissolution rate should be calculable.

The concentration gradient can be represented by $(C_s - C)/\delta$, where C_s is the concentration at saturation, C is the concentration at any time t, and δ is the Nernst-Brünner film thickness. Under existing conditions, δ could be considered constant, and C equal to zero or small compared to C_s. Therefore, if the presence of an adsorbent was to change the dissolution rate, it would have to alter C_s. An increase in solution rate would result if C_s increased, the solution rate would remain constant if C_s remained constant, and a decrease in solution rate would re-

sult if C_s became smaller. The meaning of C_s would thus explain the action of the adsorbent. If C_s is the total solubility (adsorbed and solvated molecules) of the solid undergoing dissolution in a suspension of the adsorbent, an increase in the dissolution rate, above that obtained in distilled water (dilute solution), would be expected. If C_s is the solubility of the solid in the solvent only, C_s would remain constant and the dissolution rate would not change. Since the thickness of the saturated layer next to the surface of the solid is probably only monomolecular in thickness (12), the latter reasoning might be expected to be correct. This saturated layer at the solid-water interface is part of the stagnant film at the surface of the dissolving particle.

The introduction of an adsorbent into the bulk solution could, of course, also increase the viscosity of the system. In order to maintain viscosity essentially constant it would be necessary to limit the experiment to the use of small quantities of adsorbent. Since only small quantities of adsorbent could be used, the adsorptive capacity of the adsorbent for the adsorbate would have to be great. The adsorptive capacity, of course, can be determined by means of adsorption isotherms.

The experimental method previously described by Parrott (13) was employed to determine the dissolution rates.

EXPERIMENTAL

Apparatus and Procedure.—The apparatus, tablet production, and general experimental procedure for determining the dissolution rate, except for minor alterations, were similar to those used in a previous investigation (13) in this laboratory.

Dissolution studies on benzoic acid tablets in freshly boiled distilled water were conducted under two sets of conditions: (*a*) a decreasing concentration gradient and (*b*) a constant concentration gradient.

For the experiments involving a decreasing concentration gradient, approximately 8×10^{-3} moles of benzoic acid were allowed to dissolve during the dissolution experiment.

Since the presence of an adsorbent was expected to increase the solution rate up to a maximum, this maximum being equal to the dissolution rate in distilled water when the concentration gradient was constant, it was necessary to determine the solution rate under these conditions. Appreciable build-up of the solute concentration was prevented by replacing the 2 L. of solution with distilled water after approximately 8×10^{-4} moles of benzoic acid had dissolved.

Since the introduction of an adsorbent into the bulk solution could increase the viscosity of the system, the use of small quantities of a nonhydrating and highly adsorptive adsorbent was proposed to circumvent this problem. Adsorption isotherms for benzoic acid on Norite A, were determined in the following manner: to a 125-ml. wide-mouth, ground-glass stoppered container containing 1 Gm. of Norite A, 100 ml. of a standard benzoic acid solution were added. Control runs, using distilled water in place of the benzoic acid solution were included in order to correct for the alkalinity resulting from the method of preparation of the Norite A. The containers were closed and shaken for twenty-four hours at a temperature of 25°. At the end of this time period the suspension was centrifuged and a sample of the clear supernatant fluid taken for analysis with the aid of a volumetric pipet, the tip of which was covered with glass wool. The amount of benzoic acid in solution was determined by titrating with a standard sodium hydroxide solution under an atmosphere of nitrogen, using phenolphthalein as the indicator. The amount of benzoic acid adsorbed by Norite A was calculated by difference.

The Norite A suspensions (2 L. of freshly boiled distilled water plus an accurately weighed quantity of Norite A) were prepared twenty-four hours before using. The alkalinity, introduced by the Norite A, was eliminated by titrating to pH 7.00, using a standard hydrochloric acid solution and a pH meter. In order to insure that this variable was reduced to the point of insignificance, twenty-four hours were allowed for the neutralization. Approximately 8×10^{-3} moles of benzoic acid were allowed to undergo dissolution in the suspensions.

The viscosity of each suspension was determined with an Epprecht rheometer before and after completion of each dissolution experiment.

RESULTS AND DISCUSSION

Surface-Weight and Shape-Volume Factors.—Visual inspection and the determination of the shape-volume factor to within 1% of its theoretical value showed that benzoic acid tablets maintained their shape during dissolution in both distilled water and the Norite A suspensions. The evaluation of the velocity constant with the aid of the experimentally determined surface-weight factor was, therefore, valid. The method and mathematical relationships for determining the shape-volume factor, the surface-weight factor, and the velocity constant have been described in several previous papers (13–15).

Dissolution Studies in Distilled Water.—*A Decreasing Concentration Gradient.*—In order to apply special case 2 of the Hixon-Crowell general equation (15) to the data, it was necessary to restrict the amount of benzoic acid that was dissolved in the solution. Plots of $W_0^{1/3} - W^{1/3}$ *vs.* time [see (13)] showed linearity over the concentration range studied (8×10^{-3} moles), thereby validating the use of the equation under these experimental conditions. In all subsequent experiments involving a decreasing concentration gradient, this concentration was not exceeded. The average dissolution rate was found to be 0.0425 Gm./cm.²/hr.

A Constant Concentration Gradient.—As expected, plots of $W_0^{1/3} - W^{1/3}$ *vs.* time were linear and intersected the origin. This indicated that the experimental conditions employed where an attempt was made to maintain a constant concentration gradient fell within the limits described in the derivation of the above mentioned equation, and again justified its use. The benzoic acid concentration was not allowed to exceed 8×10^{-4} moles and the dissolution rate in this case was 0.0455 Gm./cm.²/hr.

Adsorption Isotherms.—The mathematical interpretation of the adsorption data was based on the well-known Langmuir equation, $C/(x/m) = 1/AB + C/B$, where x/m is the mg. of benzoic acid adsorbed per Gm. of Norite A, C is the equilibrium concentra-

tion in mg. of benzoic acid per 100 ml. of solution, and A and B are constants. As shown in Fig. 1, the linear relationship obtained when $C/(x/v)$ was plotted against C indicated that the above equation was applicable. The adsorption capacity, B as evaluated from the reciprocal of the slope, varied between 386.1 and 406.2 mg./Gm. Since the adsorption of benzoic acid by Norite A was great, small amounts could be used which did not significantly increase the viscosity, and since the isotherms could be reproduced with reasonable precision, this substance proved to be a suitable adsorbent for the experiment.

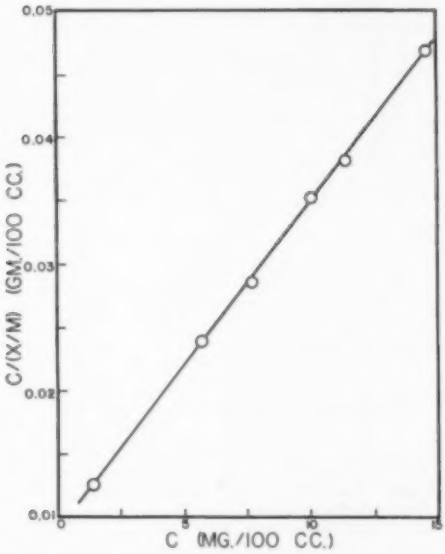

Fig. 1.—Typical adsorption isotherm for benzoic acid by Norite A.

Dissolution Studies With an Adsorbent Present in the Solvent.—The influence of the adsorbent concentration on the average dissolution rate is shown in Fig. 2. As the quantity of Norite A increased, the dissolution rate increased linearly up to a maximum. Further addition of Norite A had no influence on the dissolution rate. The maximum rate was the same as the dissolution rate in distilled water when an attempt was made to maintain the concentration gradient constant. The initial point on the graph represents the dissolution rate in water when the concentration gradient was allowed to decrease. Thus, when the concentration gradient was not constant and was not at its maximum, the addition of an adsorbent to the solution increased the dissolution rate. This occurred because the adsorbent removed drug molecules from solution which in turn prevented the concentration gradient from decreasing until the adsorption capacity was exhausted.

When the maximum dissolution rate was obtained the addition of more adsorbent to the solution did not increase the solution rate. This can be explained on the basis that the concentration gradient was at a maximum and was constant, indicating that C_s was the solubility of benzoic acid in water and not the total concentration in the suspension. If C_s was the total concentration of benzoic acid in the Norite A suspension, the concentration gradient would further increase and the dissolution rate would increase above the maximum observed rate. Also since, as previously stated, the saturated layer in the stagnant film was probably monomolecular in thickness, C_s was probably the solubility of benzoic acid in water. This might be accepted as additional proof in favor of the Nernst-Brünner film theory.

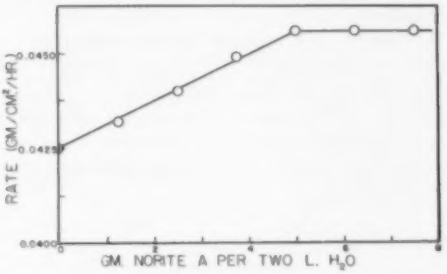

Fig. 2.—Dissolution rate of benzoic acid *vs.* concentration of Norite A.

The approximate amount of adsorbent required to increase the dissolution rate up to the maximum was calculated with the aid of the adsorption isotherms as shown in the following example. The faster rate (constant concentration gradient) in water was determined under conditions where the benzoic acid concentration was approximately 0.1 Gm. per 2 L.; whereas, with the slower rate (decreasing concentration gradient), the benzoic acid concentration was approximately 1.0 Gm. per 2 L. In order for the adsorbent to increase the rate up to the maximum, approximately 0.9 Gm. of benzoic acid would have to be adsorbed leaving approximately only 0.1 Gm. of benzoic acid actually in solution. According to the linear adsorption plot (Fig. 1), at $C = 5$ mg./100 ml. (100 mg./2 L.), the corresponding $C/(x/m)$ gave a value of 220 mg./Gm. for x/m. Therefore, if 0.9 Gm. was adsorbed, theoretically, 4.09 Gm. Norite A would be required. According to Fig. 2, approximately 5.0 Gm. of Norite A was required to give the maximum solution rate. A possible explanation for the higher value observed experimentally may lie in the fact that it was not possible in this agitated system to prevent some of the adsorbent from collecting on the walls of the vessel above the solvent level.

The pH of the suspension at the end of each dissolution experiment varied with the amount of Norite A present; the more Norite A present, the higher the final pH. This was due to the increased adsorption of benzoic acid as the Norite A concentration increased, thereby decreasing the equilibrium concentration of benzoic acid. Over the range of Norite A concentrations studied, the final pH varied from 3.60 to 4.45.

The suspensions displayed Newtonian flow and the viscosity remained nearly constant at the approximate value for water (0.98 to 1.01 c. p. s.).

SUMMARY

The influence of an adsorbent, Norite A, on the dissolution rate of benzoic acid was investigated.

The dissolution rate of benzoic acid in water was determined under conditions where no appreciable solute concentration build-up occurred and thus a nearly constant concentration gradient was maintained across the stagnant film at the solute particle surface. The rate was also determined where the solute concentration increased, resulting in a decreasing concentration gradient.

Experimental evidence indicated that an adsorbent, when used in adequate amounts, was capable of increasing the dissolution rate obtained under a decreasing concentration gradient to the maximum rate obtained at a constant concentration gradient by removing solute molecules from solution.

The approximate amount of adsorbent required to increase dissolution to the maximum rate could be calculated from the adsorption isotherm.

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Amino Acids in Opium

By A. JABBAR† and E. BROCHMANN-HANSSEN

Crude opium and fresh poppy latex have been found to contain a large number of free amino acids in addition to polypeptides liberating amino acids on hydrolysis. The relative amounts of the various amino acids differ for opium samples from several different geographic regions. This suggests that the amino acid composition may be suitable as a means of origin determination.

Since the work of Derosne and Sertürner (1), opium has been subjected to considerable study, particularly with regard to its alkaloidal composition. However, with the exception of its major alkaloids, most of the constituents of opium are still not well known, and many contradictory statements about their properties appear in the literature.

In recent years, renewed interest in the composition of opium has resulted from the opium research program of the United Nations. This research, which is carried out jointly by the United Nations Secretariat and collaborating scientists from many different countries, is directed toward developing methods for determination of the geographic origin of opium seized in illicit traffic. New interest in problems related to the biogenesis of plant constituents has also led to an increased activity in research on opium and the opium poppy.

The amino acids of opium appear to have escaped attention in spite of the fact that they probably are important building blocks in the biogenesis of the alkaloids. The relative content of opium alkaloids varies considerably, depending upon the region where the poppy is grown. It would not seem unreasonable that the amino acid composition might show a similar quantitative variation, making it useful as a basis for determination of the geographic origin of opium. This paper reports the results of a qualitative study of the amino acids in opium.

EXPERIMENTAL

Opium samples of various geographic origin were obtained from the United Nations Division of Narcotic Drugs, Geneva, Switzerland. Fresh latex was collected from opium poppies grown at the University of California School of Pharmacy.[1]

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[1] Permission to grow opium poppies, under adequate safeguards, was obtained from the Commissioner of Narcotics, U. S. Treasury Dept., Washington, D. C.

Isolation of Free Amino Acids.—Three methods were used for isolation and partial purification of the free amino acids from opium.

Lead Acetate Method.—Powdered opium was extracted with light petroleum ether by repeated maceration and decantation. After air-drying of the opium, extraction was continued with 85% ethanol. The alcoholic extracts were combined and concentrated to a small volume under reduced pressure, and organic acids, gums, and resins were precipitated with lead acetate and lead subacetate. Excess lead was removed from the filtrate by means of hydrogen sulfide. The lead sulfide was filtered off, washed free of water-soluble materials, and the combined filtrate and washings concentrated to a small volume under reduced pressure. The alkaloids were removed, first by extraction of the slightly acidic solution with chloroform, then, after addition of sodium carbonate to pH 9, by extraction with a mixture of chloroform and isopropyl alcohol (3:1). The aqueous solution was evaporated to dryness under reduced pressure and the residue extracted repeatedly with warm 95% ethanol. This alcoholic solution was concentrated and used for analysis of amino acids.

Ion Exchange Method.—To a special extraction tube (2, 3) having a coarse filter of glass wool in the stopcock were added 1 Gm. of finely powdered opium, 10 Gm. of a strongly acidic cationic exchange resin in the hydrogen form (Dowex 50-X_2, 50–100 mesh) and about 40 ml. of water. The mixture was shaken mechanically for about twenty to thirty minutes. The aqueous solution was then allowed to drain slowly from the extraction tube through an ion exchange column of Dowex 50-X_2 (H^+), 1 cm. in diameter and 20 cm. high. The extraction tube and the ion exchange column were washed with water and the adsorbed substances eluted with 1 *N* ammonia. The combined eluates were passed through a 1 × 20 cm. column of a strongly basic anionic exchange resin (Dowex 1-X_2, 50–100 mesh) previously activated with 1 *N* sodium hydroxide. After washing the column with water, the amino acids were eluted with 0.2 *N* acetic acid. The eluate was evaporated to dryness under reduced pressure and the residue analyzed for amino acids.

Acetic Acid Method.—One gram of powdered opium was triturated in a glass mortar with 4 ml. of glacial acetic acid for five to ten minutes and 20 ml. of water was added slowly while stirring. The mixture was centrifuged and the precipitate washed repeatedly by suspension in water, followed by centrifugation. The supernatant liquids were combined, evaporated to dryness under reduced pressure, and the residue analyzed for amino acids.

Amino Acids Present as Polypeptides.—Opium does not dissolve completely in glacial acetic acid at room temperature. The insoluble material was filtered off and washed, first with acetic acid, then with water. Addition of water to the solution of acetic acid-soluble material produced a flocculent precipitate which was collected and washed with water. The insoluble material and the precipitate were hydrolyzed separately by heating under reflux with 4 *N* hydrochloric acid for one hour. The solutions were filtered and the filtrates evaporated to dryness under reduced pressure. The residues were analyzed for amino acids.

Fresh Opium Poppy Latex.—Green poppy capsules were incised and the exuding latex was collected and frozen immediately in an ice-salt mixture for inactivation of enzymes. The latex was then made alkaline with ammonia and the alkaloids extracted with a mixture of chloroform and isopropyl alcohol (3:1). The amino acids in the aqueous solution were purified by adsorption on Dowex 1-X_2 (OH^-) and elution with 0.2 *N* acetic acid. The eluate was evaporated to dryness and the residue analyzed for amino acids.

Separation and Identification of Amino Acids—The amino acid mixtures were subjected to two-dimensional paper chromatography on Whatman No. 1 filter paper, $18^1/_4 \times 22^1/_2$ inches. The organic layer of a butanol-acetic acid-water mixture (5:1:4) was used in the first direction (4) and phenol-water (72:28) in the other. The papers were equilibrated for twenty-four hours in an atmosphere saturated with the solvent mixture. A 0.1% solution of ninhydrin in water-saturated butanol was used for spraying of the chromatograms. The amino acids were identified from chromatograms prepared in the same way with known amino acids and also by cochromatography of unknown with known amino acids.

Leucine and isoleucine cannot be separated by the solvent mixtures described above. However, satisfactory separation can be achieved by using a mixture of tertiary butyl alcohol, benzyl alcohol, and water in the proportions 5:5:2 as described by Thompson and Morris (5). Therefore, the spot representing leucine and/or isoleucine was eluted with water, concentrated, and rechromatographed according to Thompson and Morris alongside spots of authentic amino acids. Only isoleucine could be found in the opium samples investigated.

RESULTS

Thirteen different samples of opium, originating from seven different countries, were analyzed. In general, the amino acids present in free form were identified as follows: alanine, β-alanine, γ-aminobutyric acid, aspartic acid and its amide, asparagine, glutamic acid, glycine, homoserine, isoleucine, phenylalanine, serine, threonine, tyrosine, and valine. Three amino acids, two of which occur in relatively large amounts, still remain unidentified. Samples from various sources gave chromatograms differing in the relative amounts of the various amino acids, as observed on the basis of the size and intensity of the ninhydrin spots. Furthermore, not all amino acids listed above were detected in every sample, but this is possibly due to quantitative rather than qualitative differences. Thus, homoserine could only be identified with certainty in one sample and β-alanine in two. Valine appeared to be absent from two samples and phenylalanine from one. One sample seemed to contain β-aminoisobutyric acid instead of γ-amino-*n*-butyric acid. However, more work is needed to confirm this.

The three methods used for isolation of free amino acids from opium gave essentially the same results. The lead acetate method and the ion exchange method produced more pure samples and, therefore, better separation and sharper spots on the chromatograms. The ion exchange method was preferred because of its simplicity, and most of the work was done with this method. Although alka-

loids appear to be adsorbed quantitatively on the ion exchange resin in the extraction tube (2, 3), a second cationic exchange column was needed to achieve quantitative recovery of the acidic amino acids. This was largely due to the equilibrium condition established by the high content of strong acids in opium, specifically meconic acid and sulfuric acid. However, for qualitative identification of the amino acid content, the second ion exchange column was not necessary as sufficient amounts of even the acidic amino acids were adsorbed on the resin in the extraction tube.

Fresh poppy latex shows high enzymatic activity (6, 7). It was, therefore, conceivable that the free amino acids in opium were, at least in part, derived from enzymatic splitting of proteins during the drying of the latex. However, freshly collected and inactivated latex showed a high content of free amino acids. In addition to those mentioned under opium, proline, sarcosine, and glutamine could also be detected.

The opium fraction insoluble in glacial acetic acid gave, on hydrolysis with 4 *N* hydrochloric acid, the following amino acids: alanine, aspartic acid, glutamic acid, glycine, isoleucine, proline, serine, threonine, tyrosine, valine, and two unidentified amino acids. No amino acids were found in the hydrolysate of the precipitate produce by addition of water to a solution of opium in glacial acetic acid. Treatment of the polypeptides with Dowex 50 and warm water resulted only in very incomplete hydrolysis and liberation of small amounts of aspartic acid and glutamic acid.

DISCUSSION

Nehring and Schwerdtfeger (8) studied the composition of the protein of poppy seeds and reported the following amino acids: arginine, cystine, histidine, isoleucine and/or leucine, lysine, phenylalanine, tryptophan, and valine. It would seem, therefore, that the amino acid composition of the seed protein differs from that of opium.

Although little is known about the biosynthesis of opium alkaloids, tyrosine appears to be involved in the biogenesis of morphine (9–12). Tyrosine was found in opium although in relatively small amounts. Dihydroxyphenylalanine (DOPA) and methionine which have been said to play similar roles (9, 13) could not be detected. It is possible that DOPA, being a very unstable compound, decomposes during the isolation procedure.

A number of investigators have demonstrated a relationship between the amino acid composition of various plants and their mineral nutrition (14). Conditions which increased the total nitrogen content of potato tuber, i. e., ample nitrogen, low potassium, and phosphorus, caused an increase in the proportion of the amides, glutamine and asparagine, in the soluble nonprotein fraction (15). Arginine, and sometimes γ-aminobutyric acid, behaved in the same way. Potassium deficiency, however, reduced the γ-aminobutyric acid content to a level far below that of normal tubers.

Sulfur deficiency also produced increased levels of soluble nitrogen in mint (14) and alfalfa (16), due mainly to an accumulation of arginine, asparagine, and glutamine. Arginine could not be detected in plants grown under full nutrient supply (14).

Deficiency of certain trace elements has been found to markedly affect the composition of free amino acids in tomato plants (17). Iron and zinc deficiencies caused increased concentrations of asparagine and glutamine, while copper and manganese deficiencies resulted in increased levels of many free amino acids, but not the amides. Molybdenum-deficient plants seemed to cause reduced concentrations of total free amino acids and amides, while the β-alanine level was increased in molybdenum, zinc, and copper deficiencies. It is interesting to observe that certain amino acids, present in normal plants, could not be detected in specific deficiencies. Copper, iron, manganese, and molybdenum deficiency reduced histidine concentration to a point where it could no longer be detected. Phenylalanine could not be found in copper deficient plants, and a copper, manganese, and molybdenum deficiency had a similar effect on the lysine concentration.

Work carried out under the United Nations opium research program has indicated that there is a correlation between the mineral composition of opium and its country of origin (18, 19). Since the mineral composition of opium reflects the composition of the soil where the poppy is grown, one might expect to find a correlation between the amino acids in opium and its origin. Similar reasoning can also be based on other enviromental factors, such as temperature, light, and water supply during growth. All of these factors have been found to influence the amino acid composition of plants (14).

The work is being continued with the idea of studying the quantitative variation in the amino acid content and its relationship to the geographic origin of opium.

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) VIII

Preparation and Characterization of New Minor Alkaloids

By GORDON H. SVOBODA, MARVIN GORMAN, NORBERT NEUSS, and ALBERT J. BARNES, Jr.

Continuation of the phytochemical examination of this plant (1) which has produced two antitumor principles, vincaleukoblastine and leurosine, has yielded six previously unreported minor alkaloids in addition to those already reported from these laboratories (2). Details of the procedure leading to the preparation of isoleurosine, lochneridine, sitsirikine sulfate, vincamicine, catharine, and vindolicine, as well as their preliminary characterization, are described.

THE EXPERIMENTAL antitumor activity of vincaleukoblastine (VLB)[1] and leurosine against the P-1534 leukemia, as well as against some other transplantable neoplasms, has been reported by Johnson, Wright, Svoboda, Vlantis, and Mattas (3–6) and by Noble, Beer, and Cutts (7–13). The antimitotic activity of VLB in tissue culture has been studied by Palmer, Livengood, Warren, Simpson, and Johnson (14). The activity of these compounds in markedly inhibiting the growth of a heterologously transplanted human choriocarcinoma in the hamster cheek pouch has been shown by Hertz (15). Reports on preliminary clinical trials of VLB[2] have been presented by Hodes, Rohn, and Bond (16–19), Hertz, Lipsett, and Moy (20–22), and Warwick, Darte, and Brown (23, 24).

In addition to the 12 crystalline alkaloids already obtained from this plant in our laboratories (2)[3] continued investigation has resulted in the preparation of six new minor alkaloids. Reworking of the previously described A and B alkaloidal fractions enabled us to obtain isoleurosine and lochneridine from the former and sitsirikine sulfate and vincamicine from the latter. Vindolicine and catharine were obtained by a different procedure involving a gradient pH extraction. Alkaloids from the benzene extraction of alkalinized ammonium hydroxide drug were extracted by a dilute tartaric acid solution. The pH of this extract was adjusted to 3.3 and extracted with benzene. The crude alkaloidal mixture so obtained was chromatographed on alumina in the usual manner.

These new alkaloids are listed in Table I, along with their empirical formulas, m. p.'s, pK'a's, optical rotations, and ultraviolet absorption maxima. Their infrared spectra are reproduced separately (Fig. 1) as an additional aid to their identification.

DISCUSSION

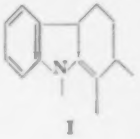
Isoleurosine.—During the crystallization of certain leurosine-containing chromatographic cuts from fraction A (2), the presence of a faster-moving component possessing an R_f value identical with that of VLB was detected by paper chromatography. Rechromatography of leurosine fractions containing this material and crystallization from methanol yielded the new unstable compound isoleurosine, tentatively formulated as $C_{46}H_{60}N_4O_9$. Its ultraviolet spectrum is extremely similar to that of leurosine and indicates the presence of indole and dihydroindole chromophores (25).

Figure 2 demonstrates the results of paper chromatographically monitoring a run of fraction A from which isoleurosine was isolated, along with leurosine and VLB. The material was chromatographed in isobutanol saturated with water on No. 1 Whatman paper saturated with pH 3.0 buffer. The paper was developed with Dragendorff's reagent in the usual manner.

A comparison of the infrared spectra of isoleurosine (Fig. 1) with that of leurosine (2, 26) clearly indicates the close structural relationship of these two compounds. The nature of this relationship is still under investigation.

Lochneridine.—Rechromatography of the post-leurosine fractions on alumina yielded lochneridine ($C_{19}H_{24}N_2O_3$) from several chloroform cuts, which crystallized as prisms from methanol.

The ultraviolet spectrum indicates a chromophore I similar to that of the alkaloid lochnericine, previously reported from this plant (27).



I

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[1] In compliance with the decision of the A.M.A. Council on drugs the name vincaleukoblastine should be replaced by vinblastine as the generic name for this alkaloid.

[2] Supplied as Velban (vinblastine sulfate, Lilly).

[3] For the sake of brevity, experimental techniques repeated from earlier work are not described in detail. A reference is given to the previous paper.

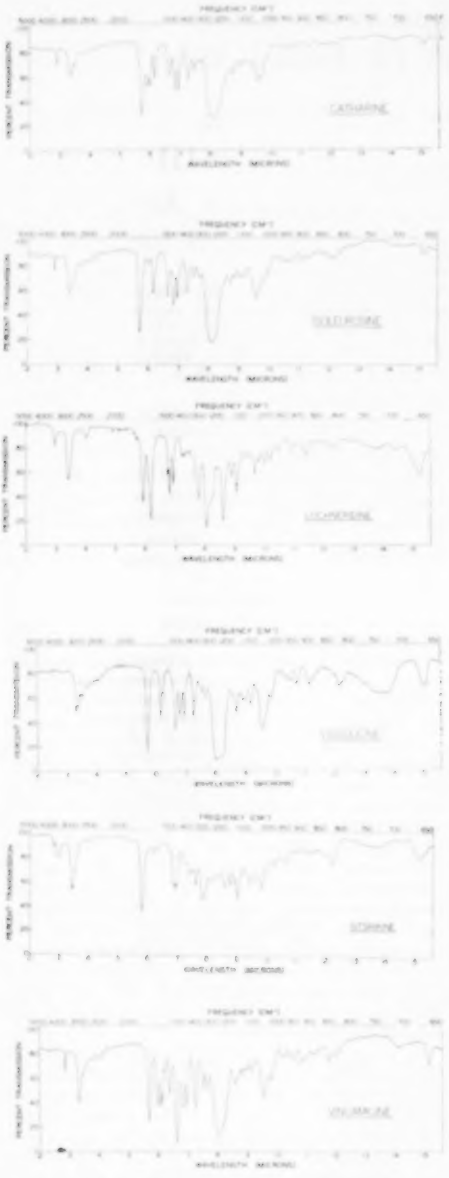


Fig. 1.—Infrared spectra of new alkaloids.

Sitsirikine.—Following the crystallization of perivine from fraction B, the mother liquor and several of the following fractions were combined and converted to the sulfate. Sitsirikine[4] sulfate ($C_{21}H_{26}N_2O_3 \cdot {}^1/_2H_2SO_4$) crystallized from ethanol, after the removal of perivine sulfate which crystallized from methanol. The free base has only been obtained as an amorphous powder.

[4] The plant from which this alkaloid was obtained was of Philippine origin; its native name is "Sitsirika."

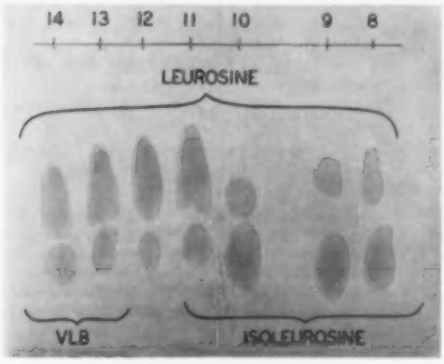
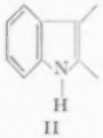


Fig. 2.—Paper chromatogram of leurosine-containing cuts of fraction A from which isoleurosine was isolated.

The ultraviolet spectrum in 95% ethanol indicates an unsubstituted indole of the general type II,



II

with an additional tertiary N in the C ring.

Titration of the sulfate in 66% DMF gave a pK'a of 7.6 for the alkaloid, with the stoichiometry and initial pH corresponding to two basic groups per sulfate ion. The equivalent weight for the salt is 406 ± 15 (or 357 ± 15 for the base).

The infrared spectrum is similar to those of certain yohimbine isomers. Comparison of the infrared spectrum of sitsirikine with that of β-yohimbine (28) shows a close structural resemblance in the region of 6.9–7.0 μ. It is therefore quite probable that sitsirikine represents a previously undescribed yohimbine isomer.

Vincamicine.—Column chromatography of a larger sample of fraction B yielded the alkaloids previously reported (2), as well as a new alkaloid vincamicine. It was isolated from the first two chloroform fractions and crystallized as needles from methanol. Analysis and electrometric titration data indicate that it is a dimeric compound, but do not allow for its unequivocal formulation.

The infrared spectrum in general resembles those of the other dimeric alkaloids obtained from this plant (2), but there is evidence of additional unsaturation or aromaticity in the 6–7 μ region.

Catharine and Vindolicine.—Chromatography of the pH 3.3 cut from the modified pH extraction produced the previously reported alkaloids catharanthine, tetrahydroserpentine, and vindoline (2) as well as the two new alkaloids catharine and vindolicine.

Catharine was obtained from several of the benzene-chloroform fractions (2:1) and crystallized from methanol ($C_{46}H_{52}N_4O_9 \cdot CH_3OH$).

The infrared spectrum of this dimeric alkaloid is similar to that of leurosine but shows evidence of additional unsaturation.

TABLE I.—NEW ALKALOIDS FROM *Vinca Rosea* Linn.

Name	Formula	M. P. °C.	$[\alpha]_D^{25}$ $CHCl_3$	pK'a in 66% DMF	U.V. $\lambda_{max.}^{EtOH}$ mμ
Isoleurosine	$C_{46}H_{60}N_4O_8$	202–206 (decompn.)	+61.2°	4.8, 7.3	214, 261, 287
Lochneridine	$C_{19}H_{24}N_2O_3$	211–214 (decompn.)	....	5.5	230, 293, 328
Sitsirikine·$^1/_2H_2SO_4$	$C_{21}H_{26}N_2O_3 \cdot {}^1/_2H_2SO_4$	239–241 (decompn.)	+23° (Base)	7.6 (Base)	224, 282, 288
Vincamicine	.....	224–228 (decompn.)	+418.0°	4.80, 5.85	214, 264, 315, 341
Catharine	$C_{48}H_{52}N_4O_9 \cdot CH_3OH$	271–275 (decompn.)	−54.2°	5.34	222, 265, 292
Vindolicine	$C_{25}H_{32}N_2O_6$	248–251 (melts, recryst.) 265–267 (decompn.)	−48.4°	5.4	212, 257, 308

TABLE II.—CHROMATOGRAPHY OF ISOLEUROSINE-CONTAINING FRACTIONS

Fraction	Eluting Solvent	Compound	Wt., Gm.	Crystallizing Solvent
1 (600 ml.)	Benzene	Oil	..	....
2–5 (5,400 ml.)	Benzene	Isoleurosine	1.97	Methanol
6–10 (2,700 ml.)	Benzene-chloroform (19:1)	Amorphous residues	..	....
11–13 (3,500 ml.)	Benzene-chloroform (3:1)	Leurosine	2.06	Methanol
14–16 (4,000 ml.)	Benzene-chloroform (1:1)	Amorphous residues	..	....
17–on (5,000 ml.)	Chloroform	Amorphous residues	..	....

TABLE III.—CHROMATOGRAPHY OF POSTLEUROSINE FRACTIONS

Fraction, 10 L. Each	Eluting Solvent	Compound	Wt., Gm.	Crystallizing Solvent
1–8	Benzene	Oil	...	....
9–11	Benzene-chloroform (3:1)	Leurosine	0.662	Methanol
12	Benzene-chloroform (3:1)	Virosine	0.049	Acetone
13–29	Benzene-chloroform (3:1)	Amorphous residues	...	....
30–32	Chloroform	Lochneridine	0.230	Acetone
33–on	Chloroform	Amorphous residues	...	....

Vindolicine ($C_{25}H_{32}N_2O_6$) was also obtained from benzene-chloroform fractions (2:1) and crystallized from ether.

The ultraviolet spectrum supports the presence of a methoxyl-substituted dihydroindole. The infrared spectrum shows it to be structurally related to vindoline (27).

EXPERIMENTAL[1]

Isoleurosine.—The leurosine-containing cuts prepared by chromatography of 200 Gm. of fraction A in the usual manner (2) produced the eight paper patterns as shown in Fig. 2.

All fractions crystallized from methanol, with the first three being particularly rich in this new alkaloid. Rechromatography of 10 Gm. of this isoleurosine-rich material in benzene on 250 Gm. of deactivated alumina gave the results listed in Table II.

Recrystallization of the isoleurosine from methanol produced blades, m. p. (loss of solvent 165°) 202–206° (decompn.) $[\alpha]_D^{25}$ + 61.2° (C = 1, $CHCl_3$).

Anal.—Calcd. for $C_{46}H_{60}N_4O_8$: C, 67.96; H, 7.43; N, 6.89; O, 17.71. Found: C, 68.19; H, 7.59, 7.70; N, 6.85; O, 17.58, 17.38.

[1] The melting points were determined on a Kofler microstage. The ultraviolet absorption spectra were obtained using a Cary, model 14, spectrophotometer, and the infrared spectra were obtained using the Perkin-Elmer, model 21, double beam, recording infrared spectrophotometer.

pK'a 4.8, 7.3, electrometric titration, 66% DMF; $\lambda_{max.}^{EtOH}$ 214 mμ (log a_M 4.70), 261 mμ (log a_M 4.21), 287 mμ (log a_M 4.09); shoulders at 296 mμ (log a_M 4.06), 310 mμ (log a_M 3.87).

Lochneridine.—A benzene solution of 82.8 Gm. of amorphous postleurosine fractions (2) was chromatographed on 3000 Gm. of alumina (Alcoa alumina, Grade F-20) deactivated with 95 ml. of 10% acetic acid in the usual manner (Table III).

The addition of acetone to the residue from fractions 30-32 (4.31 Gm.) yielded 0.230 Gm. of the crystalline alkaloid. Recrystallization from methanol gave prisms, m. p. 211–214° (decompn.).

Anal.—Calcd. for $C_{19}H_{24}N_2O_3$: C, 69.49; H, 7.37, N, 8.53; 1 OCH_3, 9.44; mol. wt., 328.4. Found: C, 69.69, 69.52; H, 6.84, 7.22; N, 7.93; 1 OCH_3, 9.30; mol. wt., 352 ± 20.

pK'a 5.5, electrometric titration, 66% DMF; $\lambda_{max.}^{EtOH}$ 230 mμ (log a_M 4.04), 293 mμ (log a_M 3.94), 328 mμ (log a_M 4.07).

Sitsirikine.—When 0.94 Gm. of the residue from the perivine mother liquors was converted to the sulfate in the usual manner, 0.083 Gm. of crystals was obtained from methanol. Recrystallization yielded 0.044 Gm. of a blue fluorescing sulfate which was identical with an authentic sample of perivine sulfate.

Evaporation of the perivine sulfate mother liquors and the addition of ethanol yielded 0.123 Gm. of a new green fluorescing sulfate. Recrystallazation

TABLE IV.—CHROMATOGRAPHY OF pH 2.8–3.3 CUT

Fraction 300 ml. Each	Eluting Solvent	Compound	Wt., Gm.	Crystallizing Solvent
1–4	Benzene	Oils	...	...
5–7	Benzene	Catharanthine (as·HCl)	1.5	Water
8–9	Benzene	Tetrahydroserpentine	0.5	Methanol
10–14	Benzene	Oils	...	...
15–20	Benzene	Vindoline	5.0	Ether
21–31	Benzene-chloroform (3:1)			
32–35	Benzene-chloroform (3:1)	Oils	...	...
36–40	Benzene-chloroform (2:1)	Catharine	0.15	Ether
41	Benzene-chloroform (2:1)	Oil	...	...
42–46	Benzene-chloroform (2:1)	Vindolicine[a]	0.07	Ether
47–on	Benzene-chloroform (2:1)	Oil	...	...

[a] Vindolicine-containing fractions can be detected by means of a bright blue color formed with Keller's reagent.

gave 0.088 Gm. of sitsirikine sulfate. While the base has not lent itself to crystallization, the sulfate crystallizes as blades, m. p. 239–241° (decompn.), $[\alpha]_D^{25}$ + 23° (free base in chloroform, C = 1).

Anal.—Calcd. for $C_{21}H_{28}N_2O_3 \cdot {}^1/_2H_2SO_4$: C, 62.51; H, 6.74; N, 6.94; O, 19.85; S, 3.97; mol. wt., 403.47. Found: C, 62.64, H, 6.81, N, 6.86, O, 20.03; S, 4.17; mol. wt. 406 ± 15.

pK'a 7.6 (base) electrometric titration, 66% DMF; $\lambda_{max.}^{EtOH}$ 224 mμ (log a_M 4.52), 282 mμ (log a_M 3.87), 288 mμ (log a_M 3.79).

Vincamicine.—Chromatography of 54.12 Gm. of fraction B (Table VI, paper V) (2) produced 9.11 Gm. of amorphous residue in fractions 28–38 by elution with chloroform. The 4.06 Gm. of residue from fractions 31–32 yielded 0.954 Gm. of crude crystalline vincamicine from methanol. Recrystallization from methanol produced needles, m. p. 224–228° (decompn.), $[\alpha]_D^{25}$ + 418.0° (C = 1, $CHCl_3$).

Anal.—Found: C, 69.48; H, 6.52, N, 8.84; O, 15.67; OCH_3, 11.58; pK'a 4.80, 5.85, electrometric titration, 66% DMF; $\lambda_{max.}^{EtOH}$ 214 mμ [log *E* (1%, 1 cm.) 2.88], 264 mμ [log E (1%, 1 cm.) 2.39], 315 mμ [log *E* (1%, 1 cm.) 2.18], 341 mμ [log *E* (1%, 1 cm.) 2.22].

Catharine and Vindolicine.—Twenty-seven Kg. of leaves were intimately mixed with 18.6 L. of water and 1.05 L. of 28% ammonium hydroxide, and this mixture was extracted with 600 L. of benzene. The percolate was concentrated *in vacuo* to 20 L. The alkaloid was extracted from this concentrate with two 35-L. portions of 2% tartaric acid. The pH of the tartaric acid extract was adjusted to pH 2.8 with ammonium hydroxide and the solution was extracted with two 20-L. portions of benzene. The pH of the aqueous phase was then adjusted to 3.3, and the solution was extracted with two 20-L. portions of benzene. When dried over sodium sulfate and concentrated *in vacuo* to dryness, the pH 2.8–3.3 cut yielded 20.4 Gm. of amorphous residue.

This residue was dissolved in benzene and chromatographed on 750 Gm. of alumina deactivated with 22.5 ml. of 10% acetic acid. These results are shown in Table IV. In addition to these two new compounds, catharanthine, tetrahydroserpentine, and vindoline were also obtained.

Catharine.—Crystallization from methanol produced orthorhombic blades, m. p. 271–275° (decompn.), $[\alpha]_D^{25}$ − 54.2° (C = 1, $CHCl_3$).

Anal.—Calcd. for $C_{46}H_{52}N_4O_9 \cdot CH_3OH$: C, 67.45; H, 6.74; N, 6.70, O, 19.12; 4 OCH_3, 14.82; OAc, 5.14; mol. wt., 836.95. Found: C, 67.45, 67.77; H, 6.95, 6.71; N, 6.97; O, 19.68, 20.02; 4 OCH_3, 15.34; OAc, 7.81 (?); mol. wt., 833.4 (X-ray).

pK'a 5.34, electrometric titration, 66% DMF; $\lambda_{max.}^{EtOH}$ 222 mμ (log a_M 4.74), 265 mμ (log a_M 4.17), 292 mμ (log a_M 4.08); shoulders at 284 mμ (log a_M 4.10) and 310 mμ (log a_M 3.95).

The hydrochloride salt was prepared in the conventional manner and was recrystallized from methanol-ether, m. p. 180–184°.

Anal.—Calcd. for $C_{46}H_{52}N_4O_9 \cdot HCl \cdot 3H_2O$: C, 61.69; H, 6.64; N, 6.26; O, 21.44; Cl, 3.96, 3 OCH_3, 10.4; (N) CH_3, 2.12. Found: C, 61.58, 61.80; H, 6.46, 6.26; N, 6.19; O, 19.52; Cl, 3.51; 3 OCH_3, 13.11; (N) CH_3, 1.68.

Vindolicine.—Crystallization from ether afforded monoclinic plates, m. p. 248–251° (melts, recryst.), 265–267° (decompn.); $[\alpha]_D^{25}$ −48.4° (C = 1, $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{32}N_2O_6$: C, 65.77, H, 7.07; N, 6.14; O, 21.03; mol. wt. 456.5. Found: C, 66.02; H, 7.29; N, 5.94; O, 21.11; mol. wt. 457.8 (X-ray).

pK'a 5.4, electrometric titration, 66% DMF; $\lambda_{max.}^{EtOH}$ 212 mμ (log a_M 4.45), 257 mμ (log a_M 3.96), 308 mμ (log a_M 3.81.)

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Urinary Excretion Kinetics for Evaluation of Drug Absorption IV

Studies with Tetracycline Absorption Enhancement Factors

By INGE O'REILLY and EINO NELSON

Absorption of plain tetracycline hydrochloride after oral ingestion by humans was compared to the absorption of this material when it was given with *d*-glucosamine hydrochloride and with citric acid. Its absorption was also compared to the absorption of tetracycline phosphate complex. Absorption evaluation was by means of excretion rate measurements. Tetracycline absorption was essentially the same from all preparations. This finding was in agreement with some findings previously reported and in conflict with others.

RECENT, exhaustive investigations have established that little or no clinically important differences exist in tetracycline blood levels obtained when tetracycline hydrochloride, tetracycline phosphate complex, tetracycline hydrochloride with glucosamine hydrochloride, or tetracycline hydrochloride with citric acid are ingested orally by normal humans (1, 2). In later studies wherein the effects of surface area and intrinsic solution rate properties of tetracycline and some of its salts were studied, it was found that no significant difference existed between maximum tetracycline excretion rates when tetracycline, tetracycline hydrochloride, and tetracycline phosphate complex were administered orally as particles having a mean diameter of about 100 μ but when using small pellets of limited surface area, substantial differences existed in maximum excretion rates (3). The various forms of tetracycline used in the first mentioned studies (1, 2) were undoubtedly powders with a mean diameter of at least 100 μ so the effect of this variable can be assumed to have been in control.

In a survey of a number of older reports on tetracycline absorption, Finland (4) has pointed out that in several cases where tests were made to determine if blood level enhancement occurred with additives, the dose of tetracycline was not controlled. In other cases, investigators chose to ignore the well-known depressing effect of calcium salts on tetracycline absorption.

In a more recent report on the effect of glucosamine hydrochloride and other materials claimed to be absorption enhancement factors, it was stated that all of the combinations mentioned earlier here gave higher blood levels than plain tetracycline hydrochloride (5). The results in this last report (5) are completely contrary in regard to effect of enhancement factors to the results given in one of the reports mentioned earlier (2) and contrary in most important aspects to the other report also mentioned earlier (1). In all three studies, the investigators were aware of the absorption depressant action of calcium salts and the necessity of controlling tetracycline potency of the dose given. There were variations in potency in both studies, but in one study (5) mean potency for all preparations was reported to be the same. In one of the other studies (2), potency differences were in several cases in favor of the compositions containing enhancement factors, yet in all cases plain tetracycline hydrochloride gave blood levels either equal to, not

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significantly less than, or greater than combinations of this material with the enhancement factors mentioned. In the remaining study (1), blood levels observed were corrected by multiplying these levels by the ratio of potency of the preparation administered to 250. The intended dose was 250 mg. tetracycline hydrochloride equivalent.

Because the results of the most recent report (5) are in conflict with earlier reports (1,2), it is of interest to report results obtained in this laboratory studying absorption of tetracycline hydrochloride, tetracycline phosphate complex, tetracycline hydrochloride with glucosamine hydrochloride, and tetracycline hydrochloride with citric acid by means of excretion rate measurements.

EXPERIMENTAL

Preparation of Dosage Forms.—Hard gelatin capsules were hand filled with the various tetracycline preparations to provide for 200 ± 5 mg. tetracycline hydrochloride activity. Four sets of capsules were used. One set contained tetracycline hydrochloride,[1] the second contained tetracycline phosphate complex, the third contained tetracycline hydrochloride and 200 mg. citric acid U.S.P., and the fourth contained tetracycline hydrochloride and 200 mg. *d*-glucosamine hydrochloride. All tetracycline materials were powders with a diameter less than 100 mμ.

Conduction of Tests.—Tetracycline preparations were ingested in the morning on fasting stomachs and no food was taken until at least one and one-half hours after drug ingestion. Prior to tests, a urine specimen was taken for blank determination. No restrictions were placed on water or food intake after the one and one-half-hour period following drug ingestion. Urine specimens were taken one, two, four, six, and eight hours after ingestion and aliquots of these refrigerated at about 1°.

A given preparation for a given subject on a particular test day was selected at random. Random selection was followed in both test series, one of which tested all four preparations during the months of June and July 1959 (series I) and the other during September and October of 1959 (series II). One week elapsed between tests on a given subject in both test series.

Assay.—The tetracycline activity of suitably diluted urines were determined in terms of the equivalent of the hydrochloride salt. The standard for tetracycline assay was the sample of hydrochloride used to prepare the dosage forms. Tetracycline activity was determined by the cylinder-plate method, using *Bacillus cereus* var. *mycoides* as the test organism (6).

Subjects.—Apparently healthy adult humans were used as test subjects. The same group of subjects were used in a given test series. The designation, ages, and weight in Kg. of the test subjects were, respectively, E-41, 77; I-34, 62; B-29, 73; P-30, 72; S-34, 57; and C-29, 57.

[1] Supplied by E. R. Squibb and Sons.

RESULTS AND DISCUSSION

Tetracycline Excretion.—Cumulative amounts of tetracycline excreted are tabulated in Table I. It is apparent that when the mean values excreted in various times found in test series I are compared, all preparations appear to be superior to plain tetracycline on first examination. However, examination of the results obtained in test series II show the direct opposite to be the case. Also, when the results obtained with a given preparation in each of the test series are averaged and the preparation averages compared, all preparations appear to be nearly the same on the basis of total excretion in a given time.

Evaluation of Results in Terms of Excretion Rate.—While the mean cumulative amounts of tetracycline excreted in a given time are a valid basis for an indirect evaluation of comparative blood levels, more direct evaluation of this quantity may be obtained by comparing excretion rates at given times from the several preparations. Mean excretion rate at a given time is directly proportional to mean blood level at the same time (7).

The mean cumulative excretion data were fitted to polynomials in powers of time of the form

$$x = at + bt^2 + ct^3 + dt^4 \qquad \text{(Eq. 1)}$$

where x is the number of mg. excreted at any time t, and a, b, c, and d constants determined by solving the set of simultaneous equations obtained by substituting for x with experimental values at one, two, four, and eight hours. This procedure is simplified by first obtaining a general solution for the coefficients of Eq. 1, using the data point times of interest.[2]

The coefficients of Eq. 1, as obtained from the averaged data of a given preparation test series mean values are given in Table II.

The derivative of Eq. 1 written for the various preparations was taken and values of excretion rate calculated at several times. These values of excretion rate were used to construct the curves shown as Figs. 1, 2, and 3. Figure 1 compares tetracycline phosphate complex to plain tetracycline hydrochloride, Fig. 2 compares tetracycline hydrochloride plus citric acid to plain tetracycline hydrochloride, and Fig. 3 compares tetracycline hydrochloride with *d*-glucosamine hydrochloride to plain tetracycline hydrochloride.

Examination of Figs. 1, 2, and 3 shows that some differences existed in maximum excretion rates from the combination of *d*-glucosamine hydrochloride and tetracycline hydrochloride and from tetracycline phosphate complex when these rates are compared to the maximum rate obtained when plain tetracycline hydrochloride was ingested. However, in

[2] When cumulative excretion data are available at one, two, four, and eight hours, the coefficients of Eq. 1 will have the following values:

$$d = (0.5\,x_8 - 7\,x_4 + 28\,x_2 - 32\,x_1)/672$$
$$c = (x_8 - 2\,x_4 - 16\,x_2 + 32\,x_1 - 3360\,d)/288$$
$$b = (x_2 - 2\,x_1 - 6c - 14d)/2$$
$$a = x_1 - b - c - d$$

where x_8, x_4, x_2, and x_1 are, respectively, cumulative excretion at eight, four, two, and one hour. Fitting data to polynomial in powers of time without a constant term implies that absorption of drug commences immediately after its ingestion. For all practical purposes, this is not an unreasonable assumption when drug is given in a rapidly dissolving form and in hard gelatin capsules as was done here.

TABLE I.—CUMULATIVE MG. TETRACYCLINE HYDROCYLORIDE EXCRETED IN VARIOUS TIMES

Dose: 200 mg. Tetracycline HCl

Subject	Test Series I 1.0	2.0	4.0	6.0	8.0	Test Series II 1.0	2.0	4.0	6.0	8.0
E	2.2	10.7	25.2	37.7	46.5	5.2	26.9	56.3	80.5	10.6
I	2.1	7.4	22.6	32.2	38.5	2.5	12.1	42.4	60.5	74.6
B	0.1	4.8	20.6	33.8	47.2	3.9	14.8	31.0	44.6	52.5
P	2.2	11.7	30.3	43.8	55.0	4.5	12.5	31.7	52.2	66.2
S	0.8	8.3	27.6	38.7	50.0	...	...	...	...	...
C	...	...	...	...	...	0.5	5.0	22.5	31.6	37.8
Mean	1.5	8.6	25.3	37.2	47.4	3.3	14.3	36.8	53.9	67.4

Dose: 200 mg. Tetracycline HCl plus; 200 mg. *d*-Glucosamine HCl

Subject	Test Series I 1.0	2.0	4.0	6.0	8.0	Test Series II 1.0	2.0	4.0	6.0	8.0
E	4.3	17.9	35.4	47.6	57.6	3.4	12.9	33.4	46.0	58.0
I	2.7	14.4	31.8	48.0	60.4	2.2	25.7	53.2	69.4	86.6
B	2.4	11.2	36.5	55.4	63.4	2.8	12.4	32.0	45.4	58.0
P	8.3	19.6	45.0	69.2	85.6	0.4	5.6	15.8	26.1	30.1
S	4.5	11.5	30.5	46.3	56.1	...	...	...	...	...
C	...	...	...	...	...	1.3	11.4	29.9	40.5	49.4
Mean	4.4	14.9	35.8	53.3	64.6	2.0	13.6	32.8	45.5	56.4

Dose: 200 mg. Tetracycline HCl; plus 200 mg. Citric Acid

Subject	Test Series I 1.0	2.0	4.0	6.0	8.0	Test Series II 1.0	2.0	4.0	6.0	8.0
E	2.2	13.4	30.4	42.2	56.5	1.2	12.8	34.3	45.3	56.3
I	2.0	11.7	30.5	45.0	56.6	0.8	8.6	39.1	61.8	75.6
B	3.0	8.4	22.6	48.2	58.8	1.2	9.1	20.5	29.6	37.0
P	4.4	11.7	34.4	44.4	49.6	2.7	12.0	27.7	39.0	48.7
S	3.0	14.8	37.5	52.8	64.0	...	...	...	...	...
C	...	...	...	...	...	3.2	9.5	25.6	35.5	43.0
Mean	2.9	12.0	31.1	46.5	57.1	1.8	10.4	29.4	42.2	52.1

Dose: 200 mg. Tetracycline HCl as Tetracycline Phosphate Complex

Subject	Test Series I 1.0	2.0	4.0	6.0	8.0	Test Series II 1.0	2.0	4.0	6.0	8.0
E	1.5	8.9	21.6	29.7	35.0	5.1	18.5	40.4	54.0	72.4
I	4.0	13.7	31.2	45.5	57.3	2.1	10.6	21.2	25.1	31.6
B	2.3	11.7	30.3	43.8	55.0	1.6	24.2	49.7	55.6	72.3
P	1.5	11.3	36.4	59.4	75.2	1.0	9.8	...	29.4	46.5
S	2.9	21.5	42.0	57.0	65.2	...	...	...	...	...
C	...	...	...	...	...	3.3	16.3	46.2	67.8	83.8
Mean	2.4	13.4	32.3	47.1	57.5	2.6	15.9	39.4	46.4	61.3

TABLE II.—COEFFICIENTS OF THE CUMULATIVE EXCRETION EQUATION

Preparation Taken	Coefficients *a*	*b*	*c*	*d*
Tetracycline HCl	−3.06	6.70	−1.32	0.0805
Tetracycline HCl plus *d*-glucosamine HCl	−3.82	8.84	−1.94	0.127
Tetracycline HCl plus citric acid	−2.91	6.52	−1.29	0.0784
Tetracycline phosphate complex	−6.30	11.13	−2.49	0.165

both cases, excretion rate from plain tetracycline hydrochloride was more sustained. Two interpretations are possible regarding the significance of these results. Tetracycline from the combination of *d*-glucosamine hydrochloride and tetracycline from tetracycline phosphate complex may have been more rapidly absorbed, causing the greater maximum excretion rates without any increase in total amount absorbed. On the other hand, the mean rate constant for tetracycline excretion, whose value depends on the intrinsic nature of this drug and the physiological state of the test subjects, may have been larger, on the average, when the subjects participated in tests using tetracycline hydrochloride with *d*-glucosamine hydrochloride and tetracycline phosphate complex. Excretion rate is proportional to the product of the excretion rate constant and body level of drug (3, 7). An increase in the constant would cause excretion rate to increase even though maximum body level was the same or nearly the same in all cases. However, excretion rate would decrease more rapidly with time in this case. Examination of Figs. 1 and 3 indicates that excretion rate did decrease more rapidly in tests with tetracycline hydrochloride and *d*-glucosamine hydrochloride and with tetracycline phosphate complex than it did in tests with plain tetracycline hydrochloride. The rapid decrease in excretion rate observed here has not been observed in other work conducted to evaluate tetracycline excretion by the same excretion rate assessment method (3).

Absorption Enhancement or Potentiation.—There is no reason to think that any one of the tetracycline preparations should be more rapidly or completely absorbed than any of the others. Absorption of many drugs may be facilitated by means such as reduction in particle size in order to increase surface

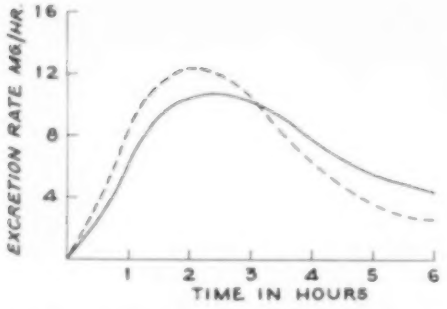

Fig. 1.—Comparison of tetracycline as the hydrochloride excretion rates when 200 mg. of plain tetracycline hydrochloride was taken by test panel (solid line) and when 200 mg. of tetracycline phosphate complex was taken by the same test panel (broken line).

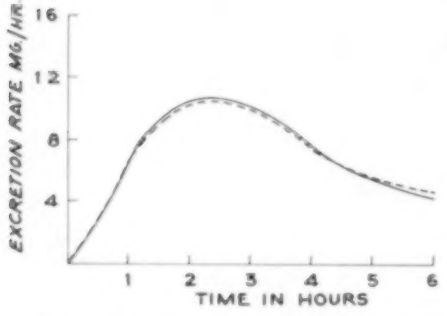

Fig. 2.—Comparison of tetracycline as the hydrochloride excretion rates when 200 mg. of plain tetracycline hydrochloride was taken by test panel (solid line) and when it was taken with 200 mg. citric acid (broken line).

area, but facilitation of absorption by this means, while important, could hardly be correctly referred to as an enhancement or a potentiation with the unknown connotation given these terms in connection with tetracycline absorption. Although several salt forms of a given drug may be absorbed at different rates because of their intrinsic solution rate properties, this factor could not be pertinent in the present work since tetracycline hydrochloride was used in all except the tests with tetracycline phosphate complex. It has been previously shown that particles of tetracycline phosphate complex having a mean diameter of 100 mμ or less are available for absorption as rapidly as tetracycline hydrochloride even though the intrinsic solution rate of tetracycline phosphate complex is less than that of tetracycline hydrochloride (3).

Some cationic drugs are known to bind to gastrointestinal mucous (8) and an interaction such as this could slow absorption rate. It remains to be established that this occurs with tetracycline and if so, that the binding could be reversed with enhancement factors. Furthermore, even if an enhancement factor reversal binding did occur, that the extent of the binding was such that measurable interference in absorption resulted.

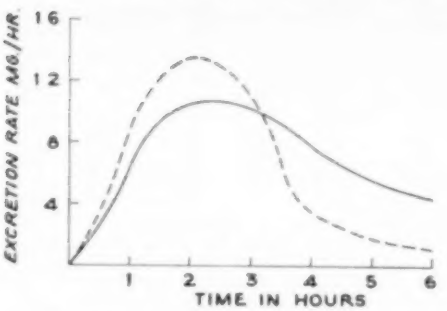

Fig. 3.—Comparison of tetracycline as the hydrochloride excretion rates when 200 mg. of plain tetracycline hydrochloride was taken by test panel (solid line) and when it was taken with 200 mg. *d*-glucosamine hydrochloride (broken line).

Tetracycline absorption has been reported to be absorbed passively (9). This fact alone indicates that absorption improvement of this drug would result through the influence of physical or chemical properties of additives. If absorption was by means of a biochemical process, additives could conceivably participate in such a process to enhance absorption, but this is not of concern when absorption is passive.

While tetracycline is known to chelate with calcium or magnesium, and addition of these materials as salts to tetracycline depresses absorption of the latter material, Boger (2) has shown that the simultaneous administration of ethylenediaminetetraacetic acid (EDTA) with tetracycline has no marked influence on its absorption. The stability constant of the calcium chelate of EDTA is much greater than the corresponding chelate with, for example, citric acid, one of the materials used as an absorption enhancement factor.

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Kinetics of Sulfamethylthiadiazole Acetylation and Excretion in Humans

By EINO NELSON and INGE O'REILLY

In studying the postabsorptive and postequilibrative phases of sulfamethylthiadiazole excretion and acetylation in humans, it was found that urinary excretion of unchanged drug and its acetylation could be described by competitive first-order processes. The excretion of acetylated drug was also by a first-order process that followed its first-order acetylation. The mean half-life for excretion of unchanged drug was about one and one-half hours, for formation of acetylated drug about fifteen hours, and for excretion of acetylated drug about one-half hour. Experimental values of excretion were in excellent agreement with values predicted by equations previously developed to describe the kinetic model found to hold in the present work.

IT WAS RECENTLY reported that the acetylation of sulfisoxazole, urinary excretion of acetylsulfisoxazole, and urinary excretion of unchanged sulfisoxazole in humans could be described by a kinetic model that consisted of two competitive first-order processes, respectively, for acetylation and excretion of unchanged drug, and a two-step consecutive first-order process, the first step of which was the formation of acetylated drug and the second the urinary excretion of this material (1). A method for evaluation of the first-order rate constants for the various processes was also described for the postabsorptive and postequilibrative phases of drug distribution in the body. This paper presents the results of a similar study made with sulfamethylthiadiazole to determine whether or not the same model could account for the excretion and acetylation of this drug.

THEORY

In the kinetic model discussed above, the excretion of acetylated drug and excretion of unchanged drug are described by the following two equations (1)

$$Sae = \frac{1}{K - k_2}\left[(KSa^0 - k_2Sa^0 + k_1S^0)(1 - \exp[-k_2t]) - \left(\frac{k_1k_2}{K}S^0\right)(1 - \exp[-Kt])\right] \quad \text{(Eq. 1)}$$

$$Se = S^0f(1 - \exp[-Kt]) \quad \text{(Eq. 2)}$$

The terms in Eqs. 1 and 2 have the following meaning: Sae = mg. acetyl sulfamethylthiadiazole excreted in any time after zero time; in order to simplify material balance this quantity is taken in terms of unchanged drug. K = the sum of the first-order rate constants for acetylation, k_1, and excretion of unchanged drug, k_2, in reciprocal hours. Sa^0 = mg. of acetylsulfamethylthiadiazole as sulfamethylthiadiazole in the body at zero time. k_2 = first-order rate constant for elimination of acetylated drug in reciprocal hours. k_1 = first-order rate constant for formation of acetylated drug in reciprocal hours. S^0 = mg. of unchanged sulfamethylthiadiazole in the body at zero time. Se = mg. of unchanged sulfamethylthiadiazole excreted in any time after zero time. f = fraction of a dose excreted as unchanged drug.

Treatment of data in terms of the kinetic model requires a shift in zero time to some time long enough after ingestion of drug to allow description of the removal and metabolism processes in the postabsorptive and postequilibrative periods for which the equations were derived. When this shift is made, cumulative excretion values are corrected for by excretion in the elapsed time period. The method of data treatment and method for deducing the various constants in Eqs. 1 and 2 has been described (1).

An interesting result may be obtained from Eq. 1 when k_2 is substantially greater than K. This equation will reduce to a simplified form after a sufficiently long enough time has elapsed to make $(1 - \exp[-k_2t]) \cong 1$. When this condition holds,

$$Sae = \frac{k_1k_2S^0}{K(k_2 - K)}(1 - \exp[-Kt]) + A \quad \text{(Eq. 3)}$$

where A is a constant. Depending on how much larger k_2 is than K, Eq 3 may describe excretion of unchanged drug from the time taken as zero or after some time subsequent to this. Assuming that the latter case holds, and another shift in zero time is made, and the experimental values of excretion in times following are corrected by subtracting the amount of acetylated drug excreted to the new zero time, then

$$Sa'e = Sa_\infty'(1 - \exp[-Kt']) \quad \text{(Eq. 4)}$$

where $Sa'e$ is the amount excreted after this new zero time and Sa_∞' is the total amount of acetylated drug excreted minus the value of Sae from excretion to the new zero time as obtained experimentally. The value of time, t', is clock time minus the elapsed time to the new zero time. If $\log(1 - Sa'e/Sa_\infty')$ is plotted *vs.* time, the value at K may be obtained from the slope and compared to the same quantity obtained from a similar plot based on Eq. 2 which describes excretion of unchanged drug.

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EXPERIMENTAL

Adult humans in apparent normal health ingested 1-Gm. doses of powdered sulfamethylthiadiazole suspended in approximately 200 ml. of water in the morning on fasting stomachs. Urine specimens were collected at appropriate times, volume noted, and aliquots placed under refrigeration at about 2° until assayed. Specimens were assayed for free and total sulfonamide by the Brattan and Marshall procedure. Appropriate blank corrections were made. Data on the subjects are given in Table I.

TABLE I.—DATA ON SUBJECTS PARTICIPATING IN EXCRETION TESTS

Initial	Weight, Kg.	Age, yr.	Sex
E	77	39	M
S	57	33	F
I	61	33	F
N	77	21	M
K	68	28	M
B	68	28	M

RESULTS AND DISCUSSION

Cumulative amounts of free and acetylated sulfamethylthiadiazole excreted to various times are given in Table II. Extent of acetylation and excretion velocity of free drug appear to be in agreement with observations previously reported (2).

Initial calculations indicated that zero time for the purposes of calculating the various constants in Eqs. 1 and 2 by the method described previously could be taken as one and one-half hours after drug administration (1). Rate and other constants needed to describe the excretion of free and acetylated drug by Eqs. 1 and 2 for each of the subjects are given in Table III. In several cases extent of acetylation was too small to allow determination of k_2. The agreement between values of excretion calculated by Eq. 1 for acetylated drug and experimentally determined values for most of the subjects is shown in Fig. 1. A similar comparison for unchanged drug is shown in Fig. 2. One curve in Fig. 1 was drawn after interpreting excretion data following a 2.0-Gm. dose of sulfamethylthiadiazole intravenously (2). Zero time in this case was one hour after drug administration. It appeared that the model previously shown to describe sulfisoxazole excretion and acetylation was capable of accounting for acetylation and excretion of sulfamethylthiadiazole (1).

The mean value for fraction of dose excreted as free drug was relatively large, but the rate constant for formation of acetylated drug was not insignificant. The mean value for this latter constant, k_1, was about 0.046 hr.$^{-1}$ which corresponds to a half-life for the process of about fifteen hours ($t_{1/2} = 0.693/k$). For comparison, sulfisoxazole, which was recovered in the urine in a considerably greater amount as acetylated drug, had a mean half-life for the acetyla-

TABLE II.—CUMULATIVE MG. FREE (F) AND ACETYLATED (A) SULFAMETHYLTHIADIAZOLE AND ML. URINE EXCRETED BY TEST SUBJECTS IN VARIOUS TIMES FOLLOWING ORAL INGESTION OF 1.0-GM. DOSES OF DRUG IN WATER SUPSENSION[a]

	mg. Excreted															
	0.5		1.5		2.5		3.5		6.0		8.0		12.0		24.0	
Subject	F	A	F	A	F	A	F	A	F	A	F	A	F	A	F	A
E	40	1	256	33	475	74	645	75	791	103	842	103	868	103	876	103
S	75	25	302	50	504	112	609	163	749	219	781	226	797	236	802	239
I	76	0	532	0	759	6	861	9	994	9	1026	9	1040	9	1043	9
N	26	0	374	0	611	0	759	7	913	15	963	24	986	44	990	53
K	72	0	600	0	770	0	889	0	1006	0	1034	0	1062	0	1069	0
B	36	9	509	82	704	97	767	125	855	146	876	156	889	157	893	157

	ml. Urine						
Subject	0.5	1.5	2.5	3.5	6.0	8.0	12.0	24.0
E	70	350	390	450	670	930	1910	2310
S	50	100	190	250	530	670	1000	1470
I	140	310	450	510	670	810	990	1450
N	90	330	420	520	630	710	960	1550
K	20	160	190	230	420	480	700	1110
B	30	90	150	210	420	580	1290	1620

[a] Excretion of acetylated drug is given in terms of unchanged drug.

TABLE III.—RATE AND OTHER CONSTANTS INVOLVED IN SULFAMETHYLTHIADIAZOLE ACETYLATION AND EXCRETION BY TEST SUBJECTS

	Subject						
Constant	E	S	I	N	K	B	43[c]
k_1 (hr.$^{-1}$)	0.047	0.114	0.005	0.024	0	0.083	0.048
k_2 (hr.$^{-1}$)[a]	3.0[b]	1.74 (0.017)	...	0.148 (0.018)	...	1.15 (0.14)	1.31 (0.060)
k_3 (hr.$^{-1}$)	0.400	0.381	0.508	0.438	0.513	0.472	0.565
K (hr.$^{-1}$)	0.447	0.495	0.513	0.462	0.513	0.555	0.613
S^0 (mg.)	692	650	515	650	469	452	1000
Sa^0 (mg.)	0	39	0	19	0	7	81
f	0.896	0.770	0.991	0.948	1.00	0.850	0.920

[a] Shown with standard error in brackets. [b] Approximate value. [c] Constants calculated from data previously published for excretion following a 2.0-Gm. intravenous dose (2).

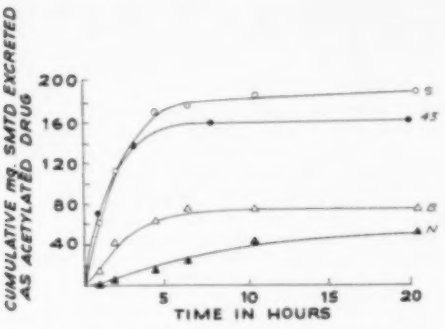

Fig. 1.—Cumulative excretion curves for acetylsulfamethylthiadiazole. The lines show theoretical excretion and the plotted points are experimentally determined values. Excreted amounts shown are from the time taken as zero and are given in terms of unchanged drug. Curves are labeled with subjects' identification.

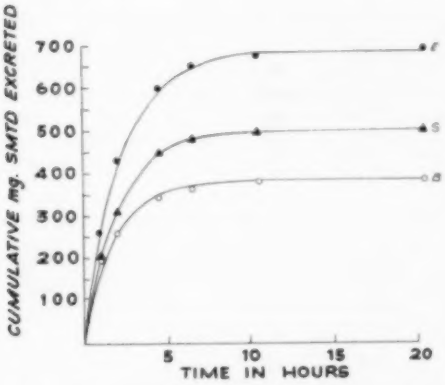

Fig. 2.—Cumulative excretion curves for free sulfamethylthiadiazole. The lines show theoretical excretion and the plotted points are experimentally determined values. Excreted amounts shown are from time taken as zero. Curves are labeled with subjects' identification.

tion process of about 30 hours (1). The results are a consequence of the competitive nature of the processes for excretion of free drug and for acetylation. The process for excretion of unchanged sulfamethylthiadiazole had a mean half-life of about one and one-half hours. The half-life for the same process with sulfisoxazole was about seven hours and fifty-four minutes. The kinetic basis for these results may be seen from the following discussion: the fraction of a dose, f, of a sulfonamide recovered in urine as unchanged drug at a time essentially infinity in terms of the experiment bears the following relationship to the rate constant for elimination of free drug, k_2, and the rate constant for acetylation, k_1 (1)

$$f = \frac{k_2}{k_1 + k_2} \qquad \text{(Eq. 5)}$$

The increase in extent of acetylation of sulfisoxazole as compared to sulfamethylthiadiazole was due to k_2 being smaller for sulfisoxazole than it was for sulfamethylthiadiazole. The difference in the values of this quantity overrode the difference in k_1 which was such as to have caused less sulfisoxazole to be excreted as acetylated material if k_2 had the same value with both compounds.

From the preceding, it may be seen that it is important to determine the value of pertinent constants rather than to base conclusions on gross observation as to the extent of a biotransformation. For example, it was recently reported by Campbell and coworkers (3) that exposure to cold decreased the extent of acetylation of *p*-aminobenzoic acid by rats. This conclusion was based on the relative amounts of free and acetylated *p*-aminobenzoic acid found in the urine after dosage under two conditions of environment. A conclusion to the effect that exposure to cold increased urinary excretion rate of free drug has the same validity as the one reported because of the considerations just discussed.

A comparison of the ratio of the rate constant for excretion of acetylated drug (k_1) to the rate constant for excretion of unchanged drug (k_2) in the cases of sulfamethylthiadiazole and sulfisoxazole is of interest. With sulfisoxazole, the ratio had a value of about 0.9. With sulfamethylthiadiazole, the value of the same ratio was about 3. In the latter case, in spite of the same general type biochemical transformation, the metabolite, acetylsulfamethylthiadiazole was more rather than less rapidly cleared by the kidney. However, with one subject (N), this ratio was less than one.

Sufficient data were available on one subject (S) to allow test of Eq. 4. The rate constant for overall removal of sulfamethylthiadiazole from the body deduced from the plot described in connection with Eq. 4 (shown as Fig. 3) was 0.44 hr.$^{-1}$. This compares to the value of about 0.5 hr.$^{-1}$ (Table III) obtained by following excretion of unchanged drug. The shift in zero time necessary to make the plot of Fig. 3 was two and one-half hours from the time of drug ingestion.

Excretion rate of acetylated drug is given by the derivative of Eq. 1. Thus

$$\frac{dSae}{dt} = \frac{k_2}{K - k_2}\left[(KSa^0 - k_2Sa^0 + k_1S^0)(\exp[-k_2t]) - (k_1S^0)(\exp[-Kt])\right] \qquad \text{(Eq. 6)}$$

The time of maximum rate of excretion of acetylated drug, $t_{max.}$, will be given by solving for time after taking the derivative of Eq. 6 and equating the resulting expression to zero. By this operation

$$t_{max.} = \frac{2.30 \log [k_1KS^0]}{K - k_2} - \frac{2.30 \log [k_2Sa^0(K - k_2) + k_1k_2S^0]}{K - k_2} \qquad \text{(Eq. 7)}$$

Since the amount of acetylated drug Sa, in the body at any time is given by (1)

$$Sa = \frac{1}{k_2}\frac{dSae}{dt} \qquad \text{(Eq. 8)}$$

determination of $t_{max.}$ for substitution into Eq. 6 to calculate $(Sa)_{max.}$ by Eq. 8 is of some interest. For example, it is easily calculated from data in Table III using Eq. 7 that maximum excretion rate of acetylated drug occurred in subject E at three-

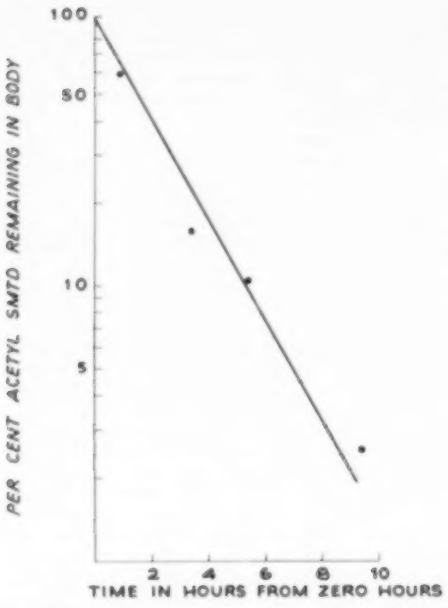

Fig. 3.—Relationship between acetylsulfamethylthiadiazole remaining to be excreted and time after a further shift in zero time and adjustment of excreted amounts by cumulative excretion to the new zero time.

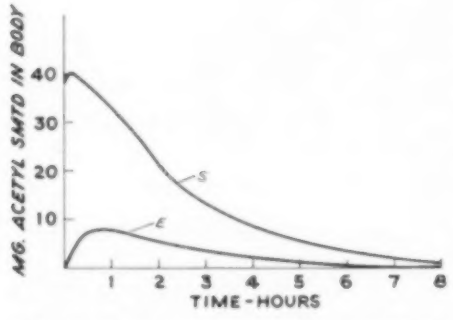

Fig. 4.—Body level of acetylated sulfamethylthiadiazole as a function of time in two subjects. Times shown are after zero time shift. Curves are labeled with subjects' identification.

fourths of an hour after the time taken as zero time or two hours and fifteen minutes after drug ingestion. The rate at this time was from Eq. 6, about 23 mg./hour. From Eq. 8 the maximum amount of acetylated drug in the body of this subject was about 8 mg. This small value is consistent with the large value found for the constant, k_2, which determines removal rate of acetylated drug. Results of calculating the same quantities for some of the other subjects are summarized in Table IV.

Table IV.—Excretion Rate and Amount of Acetylated Drug in Body at $t_{max.}$ for Several Test Subjects

Subject	$t_{max.}$, Hours after 0 Time	Maximum Excretion Rate, mg./hr.	Maximum Amount Acetylated Drug in Body, mg.
E	0.75	23	8
S	0.15	70	40
N	2.58	5.3	36
B	1.05	22	19

Equations 6 and 8 may be used to calculate body levels of acetylated drug at any time and results of these calculations are shown in Fig. 4 for subjects E and S. The maxima of these curves correspond to the maxima just calculated, and not to maxima resulting from continuing absorption of the precursor, sulfamethylthiadiazole, since all data were from post absorptive phases of drug distribution and metabolism.

SUMMARY

A kinetic model previously shown to describe the postabsorptive and postequilibrative phases of sulfisoxazole excretion and acetylation in humans, as well as excretion of the acetylated drug, was tested for its ability to describe the same processes for sulfamethylthiadiazole. The integrated mathematical expressions describing the kinetic model with constants evaluated for experimental values of sulfamethylthiadiazole excretion were capable of accounting for the respective processes with this drug.

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Effects of Iproniazid on *Tetrahymena pyriformis*

By JOSEPH Z. KREZANOSKI

The effect of iproniazid on the rate of increase of cell mass and on the respiration of the cells and homogenates of *Tetrahymena pyriformis* has been studied for the purpose of obtaining information concerning the biological action of this drug. A number of different substrates were employed in the respiration studies. Among these, L-tyrosine had a marked stimulating effect on the respiration in both the cells and homogenates. In the presence of L-tyrosine there was a twofold increase in the drug-produced depression of homogenate respiration. Tyramine was not metabolized and had no effect on the drug-produced depression of respiration.

THE COMPOUND, iproniazid (1-isonicotinyl-2-isopropylhydrazine), was synthesized (1) as a variant of isoniazid (isonicotinic acid hydrazide) for its possible use in the treatment of tuberculosis. It was found that iproniazid was indeed effective in the treatment of tuberculosis (2); but in addition, it displayed many other interesting effects. Among these effects are: central nervous stimulation (3), potentiation of the effects of other drugs (4), hepatitis (5), beneficial action on angina pectoris (6), hypotensive action (7), favorable results in treating acne vulgaris (8), and beneficial effects in treating rheumatoid arthritis (9). In 1952 Zeller and Barsky (10) found that iproniazid strongly inhibits monoamine oxidase in a variety of biological preparations. This effect has been verified by many investigators and has been proposed as the mode of action. In support of this theory ample evidence has been presented to show that metabolites, such as certain catecholamines and serotonin, do accumulate in biological systems in the presence of the drug (11). Although some of the clinical effects can be readily accounted for on the basis of an irreversible inhibition of monoamine oxidase and an accumulation of serotonin and catecholamines, it does not appear to be a likely explanation in all instances (12).

The protozoan *Tetrahymena* has been employed successfully in studies concerned with the mode of action of cellular depressants (13–18); in these studies attention is given to the possible effects of a potential stimulant on this organism. *Tetrahymena* are rather uniquely suited for pharmacological and metabolic studies because of ease of culture, rapid growth, ready availability of large masses of cells, as well as for other reasons (19). This report describes experiments designed to assess the effect of iproniazid on the rate of increase of cell mass, the endogenous respiration of cells and homogenates, as well as on the respiration of cells and homogenates to which certain substrates have been added.

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MATERIALS AND METHODS

Culture Techniques.—A sterile stock culture of *Tetrahymena pyriformis* W was maintained in the dark at 28 ± 1° in 300-ml. Erlenmeyer flasks containing 15 ml. of medium consisting of 1.8% proteose-peptone (Difco) and 0.2% yeast extract (Difco). Transfers were made every three to four days. In those experiments where large numbers of cells were required for respiration studies, the inoculation was made into 2,000-ml. Erlenmeyer flasks containing 400 ml. of medium. The cells were harvested at the end of seventy-two hours by centrifugation for three minutes in an angle head centrifuge at 150 × g. The cells were washed three times with deionized water and then suspended in 0.037 *M* phosphate buffer (pH 6.9).

Growth Studies.—All growth studies were carried out at 28° in 300-ml. Erlenmeyer flasks fitted with side arm tubes (Bellco Nephelo-Culture flasks) suitable for insertion into a Klett-Summerson instrument. The medium for these studies was prepared as a concentrate so that it could subsequently be diluted by adding variable amounts of a concentrated iproniazid solution and deionized water to yield a final concentration of 1.8% proteose-peptone and 0.2% yeast extract. In each instance the flasks contained 15 ml. of medium, with or without iproniazid, and the inoculum consisted of 0.1 ml. of a twenty-four hour log phase culture. The rate of increase in cell mass was followed by taking readings in a Klett-Summerson photoelectric colorimeter approximately every twelve hours, or oftener; the turbidity values are reported in Klett units. Uninoculated medium served as a blank and all determinations were made in triplicate.

Respiration Studies on Cells.—The effect of various concentrations of iproniazid on the rate of respiration was determined at 28° using conventional Warburg techniques. The Warburg vessels were loaded with 1.0 ml. of a cell suspension containing about 3.0 mg. of cell protein (20) to which was added 0.5 ml. of an appropriate concentration of iproniazid phosphate made up in 0.037 *M* phosphate buffer and adjusted to pH 6.9. Additional phosphate buffer, with or without substrates, was added to give a final volume of 2.3 ml. in the main com-

partment. The center well contained 0.2 ml. of 20% potassium hydroxide and filter paper. Respiration experiments were carried out after a ten-minute equilibration period with air serving as the gas phase and a shaking rate of 120 excursions per minute. Because of a delay in the onset of drug action, as pointed out later, respiratory data were collected during the second thirty-minute period following temperature equilibration.

Respiration Studies on Homogenates.—In these studies the washed cells were suspended in ice-cold phosphate buffer (0.050 *M* and pH 6.9) containing sufficient sucrose to yield a final concentration of 0.25 *M*. The suspension was passed through a pharmaceutical hand homogenizer twice without delay and from then on, the homogenate was maintained at 0° until it was loaded into the Warburg vessels. The first thirty-minute respiration values were used in all the homogenate respiration data presented here. Best results were obtained when the final volume of 2.3 ml. in the main compartment contained 10 mg. of *Tetrahymena* protein per ml. In all instances wherein substrates were added the pH of the substrate solution was adjusted to 6.9.

Reagents.—All the reagents were used in these experiments without further purification. In each instance an effort was made to obtain reagents of the highest purity, including iproniazid phosphate (Hoffmann-LaRoche, Inc., obtained as Marsilid phosphate), tyramine hydrochloride (Eastman), and L-tyrosine (Eastman).

RESULTS AND DISCUSSION

The effect of different concentrations of iproniazid on the rate of increase of cell mass is presented in Fig. 1. For purposes of ease of presentation only three different concentrations of the drug are shown in the figure; however, concentrations as low as 1×10^{-6} *M* were studied. These lower concentrations were studied to see if they would have any stimulating effect on the increase of cell mass. In some experiments there was some small stimulation; but this effect was not consistent in repeated experiments indicating that perhaps this finding was without significance. It may be noted that concentrations of iproniazid above 3×10^{-4} *M* completely arrest the increase in cell mass while lesser concentrations have a marked effect on the lag phase and the log phase, as well as on the maximum populations attained.

In attempting to study the effect of iproniazid on the respiration of intact cells it became apparent that the degree of inhibition was dependent upon the time of incubation. Figure 2 represents the results of one such experiment designed to establish the incubation time necessary to achieve the full effect of the drug. The values presented in the graph are averages of duplicate vessels, each containing 2×10^{-3} *M* iproniazid in addition to the components described earlier. From these and other data obtained using different concentrations of the drug it was concluded that at least thirty minutes of incubation was necessary for iproniazid to elicit its full inhibitory effect on cell respiration. Since this delay of onset of depression of respiration did not occur in homogenates, it was reasoned that iproniazid penetrates the cell membrane with difficulty and time is necessary for complete equilibration. As a result of this finding a thirty-minute incubation period was allowed in all whole cell respiration studies reported here.

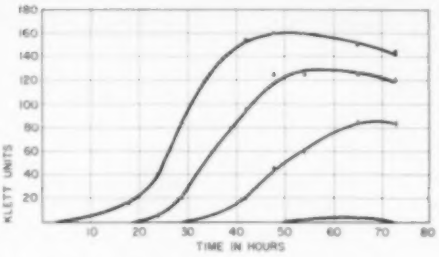

Fig. 1.—The effect of iproniazid on the rate of increase of cell mass. ● control; ▲ 2×10^{-3} *M*; ◆ 3×10^{-3} *M*; ■ 4×10^{-3} *M*.

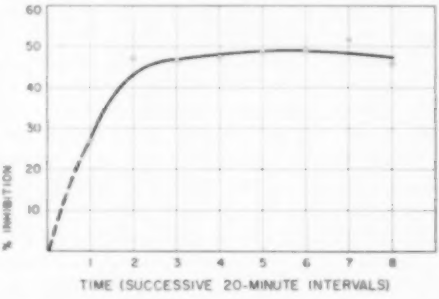

Fig. 2.—The effect of incubation time on the inhibition of intact cell respiration by 2×10^{-3} *M* iproniazid. The respiration values used in calculating per cent inhibition are the values for control and experimental vessels obtained at the end of each successive twenty-minute interval.

Table I.—Effect of Iproniazid on Endogenous Whole Cell Respiration

Iproniazid Concn. × 10^{-3} *M*	Per Cent Inhibition	Number of Duplicate Determinations
2.5	8.5 ± 1.08[a]	4
5.0	15.0 ± 1.70	8
10.0	24.9 ± 1.55	10
20.0	46.5 ± 1.89	4
30.0	69.5 ± 1.18	7

[a] Standard error.

Data showing the effect of varying concentrations of iproniazid on endogenous whole cell respiration are presented in Table I. The per cent inhibition was calculated by comparing the respiration in the presence of iproniazid with control values. Although the lowest concentration of iproniazid shown in Table I is 2.5×10^{-3}, much effort was exerted in an attempt to observe a possible stimulating effect at lower concentrations. No clear-cut stimulation of respiration was observed with concentrations as low as 1×10^{-6} *M*.

It should be noted that a two-hour incubation of the cells in the presence of iproniazid at concentrations above 2×10^{-3} *M* caused all the cells to die. In comparing the effect of 2.5×10^{-3} *M* iproniazid

on respiration of intact cells with its effect on the increase of cell mass it may be observed that an 8.5% inhibition of respiration is associated with about a 30% reduction in the maximum population attained (Fig. 1).

In several experiments, 1×10^{-2} *M* tyramine hydrochloride was added to cell suspensions to see if the addition of this commonly employed monoamine oxidase substrate would influence the depressant effect of iproniazid (2.5×10^{-3} *M* to 30×10^{-3} *M* final concentration) on the respiration. Tyramine did not have any apparent effect.

Data were then accumulated to ascertain whether or not 0.01 *M* tyramine influenced the depressing effect of iproniazid on the respiration of homogenates.

Table II presents the results of the effect of several concentrations of iproniazid on the endogenous respiration of homogenate; Table III presents the effect of iproniazid on homogenate respiration in the presence of 0.01 *M* tyramine.

TABLE II.—EFFECT OF IPRONIAZID ON ENDOGENOUS HOMOGENATE RESPIRATION

Iproniazid Concn. $\times 10^{-3}$	Per Cent Inhibition	Number of Duplicate Determinations
5.0	5.3 ± 1.6[a]	4
10.0	14.6 ± 0.95	5
20.0	31.0 ± 1.39	4
30.0	47.3 ± 4.15	5

[a] Standard error.

TABLE III.—EFFECT OF IPRONIAZID ON HOMOGENATE RESPIRATION IN THE PRESENCE OF 0.01*M* TYRAMINE HYDROCHLORIDE

Iproniazid Concn. $\times 10^{-3}$	Per Cent Inhibition	Number of Duplicate Determinations
5.0	5.8 ± 1.9[a]	3
7.5	8.0 ± 2.2	3
10.0	13.4 ± 2.48	3
15.0	24.3 ± 1.03	4
20.0	32.8 ± 1.86	3

[a] Standard error.

In comparing the results presented in the two Tables it becomes apparent that the degree of inhibition in the two cases is about the same. If tyramine is oxidized by the homogenate and if this oxidation is mediated by an iproniazid susceptible monoamine oxidase, a change in the respiratory depression, caused by iproniazid, may have been expected. The addition of 0.01 *M* tyramine alone caused about a 10% reduction in the rate of respiration in comparison with controls.

The question arose as to whether or not *Tetrahymena* contain an enzyme capable of catalyzing the oxidation of tyramine. In order to test for the metabolic breakdown of tyramine, six duplicate sets of Warburg flasks were charged with 6.8 mg. of cell protein (added as a cell suspension). Four of the duplicate sets were charged with tyramine hydrochloride in a final volume of 2.3 ml. in the main compartment to give a final concentration of 0.01 *M*. One milliliter of 30% trichloroacetic acid was added to a set containing the added tyramine at the beginning of the experiment. These vessels served as blank and control, respectively. The remainder of the three sets of tyramine-containing flasks and one set without tyramine were shaken in the Warburg bath at 28°. The reaction was stopped with trichloroacetic acid by removing one set at a time after an incubation time of thirty, sixty, and ninety minutes, respectively. The one remaining set without tyramine was incubated for ninety minutes and served as an additional control. In analyzing for tyramine, according to the method of Udenfriend and Cooper (21), the finding was that even after ninety minutes, the tyramine was not metabolized. A similar experiment employing 11.3 mg. protein per reaction flask (added as the homogenate) gave almost identical results. Based on the findings in these two experiments, it appears that tyramine is not metabolized by *Tetrahymena* and that iproniazid does not exert its depressing effect through influencing the metabolism of tyramine. In this connection, it should be pointed out that no evidence was obtained for the presence of serotonin in *Tetrahymena pyriformis* using the technique of Bogdanski, *et al.* (22), which in our hands was used successfully to measure serotonin in rat brain.

In comparing the respiratory inhibitory effects of iproniazid on whole cells (Table I) and homogenates (Table II) it may be seen that the inhibitory effect is about twice as pronounced on whole cell respiration. In making this comparison it should be mentioned that the rate (μL. O_2/hour/mg. protein) of endogenous whole cell respiration in control flasks amounted to 35, while that of homogenates amounted to 7.

To test the possibility that a metabolic precursor of tyramine may influence the inhibition of respiration produced by iproniazid, L-tyrosine (0.01 *M*) was added to both whole cells and homogenates. It was found that in both instances the respiratory rate was increased about 50%. This finding appeared to have promise since apparently, this amino acid was metabolized by *Tetrahymena* at an unusually rapid rate.

The effect of iproniazid was re-examined on both whole cell and homogenate respiration in the presence of L-tyrosine. Table IV shows the effect of L-tyrosine on the degree of inhibition produced by iproniazid on whole cells.

Table V shows the effect of L-tyrosine on the degree of inhibition produced by iproniazid on homogenates.

In examining the effect of iproniazid on whole cell and homogenate respiration in the presence of L-tyrosine it may be seen that the inhibition differences noted in endogenous respiration studies have been eliminated. Secondly, it can be seen that there was a marked increase in the inhibitory effects of iproniazid when L-tyrosine was added. These data suggest that perhaps a tyrosinase enzyme present in *Tetrahymena* is at least one of the focal points of inhibition by iproniazid. In this connection it should be noted that by the addition of L-tyrosine it has been possible to produce a 6.1% respiratory inhibition with a 2.5×10^{-3} *M* concentration of iproniazid which also produces only a minor effect on the rate of increase in cell mass (Fig. 1). The extent of inhibition of the two parameters is not too different.

In view of the reported requirements of ascorbic acid and copper for tyrosine oxidation (23) these were added in 10^{-3} and 10^{-5} molar amounts, respec-

TABLE IV.—EFFECT OF IPRONIAZID ON WHOLE CELL RESPIRATION IN THE PRESENCE OF 0.01 *M* L-TYROSINE

Iproniazid Concn. × 10^{-3}	Per Cent Inhibition	Number of Duplicate Determinations
2.5	6.1 ± 0.57[a]	3
5.0	14.4 ± 0.57	4
7.5	23.7 ± 0.82	3
10.0	33.2 ± 1.17	3

[a] Standard error.

TABLE V.—EFFECT OF IPRONIAZID ON HOMOGENATE RESPIRATION IN THE PRESENCE OF 0.01 *M* L-TYROSINE

Iproniazid Concn. × 10^{-3}	Per Cent Inhibition	Number of Duplicate Determinations
2.5	8.3 ± 1.7[a]	2
5.0	15.1 ± 0.91	3
7.5	21.9 ± 1.77	3
10.0	33.5 ± 1.25	3

[a] Standard error.

tively, to tyrosine-containing cell suspensions and homogenates. There was no apparent increase in the respiratory rate in either case. Furthermore, the inhibitory effect of iproniazid was not modified.

Because of the chemical similarity between isoniazid and two vitamins, namely pyridioxine and nicotinic acid, investigators looked for and found that indeed some of the untoward effects of isoniazid could be reduced by the administration of these two vitamins (24–26). The possibility of a chemical relation between iproniazid and the two vitamins is apparent. The next step was to test the effect of pyridoxine and nicotinic acid on the respiratory inhibition produced by iproniazid.

In a series of experiments the effect of iproniazid on whole cell and homogenate respiration was studied in the presence of varying amounts of niacinamide (1×10^{-2} *M* to 3×10^{-2} *M*). The addition of niacinamide to homogenates in the presence as well as in the absence of iproniazid produced no apparent effect. The highest concentration of niacinamide did depress whole cell respiration, but only by about 10%.

A similar series of experiments was carried out to note if pyridoxine (1×10^{-4} *M* to 1×10^{-2} *M*) would elicit any effect on the iproniazid inhibition of respiration in whole cells as well as homogenates. The lower concentrations were entirely without effect; however, as the concentrations were increased, pyridoxine produced an inhibitory effect on respiration almost exactly equal to that of iproniazid in equal concentrations. In several experiments it was also found that the respiratory depressant effects of iproniazid and pyridoxine were additive. That is to say, that the per cent inhibition produced by 2×10^{-3} *M* of either iproniazid or pyridoxine alone was almost identical to the per cent inhibition produced by 1×10^{-2} *M* iproniazid and 1×10^{-2} *M* pyridoxine added together. These findings were true for both the whole cells and homogenates. It was reported recently that niacin and pyridoxine are without effect on iproniazid induced toxicity symptoms in mice (27). The findings reported here on *Tetrahymena* are in accord.

The results of these studies suggest that iproniazid has a biological effect which goes beyond the inhibition of a monoamine oxidase. Indeed, the toxic symptoms produced by the drug may be due to the inhibition of other enzymes, including the inhibition of oxidation of L-tyrosine. Since tyrosine is a metabolic precursor of certain catecholamines, the effect on tyrosine oxidation may be of significance.

The effect of iproniazid on the rate of cell division of *Tetrahymena* is in general agreement with the subacute, acute, and chronic toxicity data reported by others (28) obtaining their results on a number of different mammals. Perhaps, *Tetrahymena* could be employed industrially for preliminary toxicity screening tests.

SUMMARY

1. A study of the effect of iproniazid on the rate of increase of cell mass of *Tetrahymena pyriformis* shows that increase in cell mass is completely inhibited by concentrations between 3×10^{-2} and 3×10^{-4} *M*.

2. The effect of iproniazid on the respiration of whole cells and homogenates respiring endogenously as well as in the presence of tyramine and tyrosine is described.

3. The conclusion is reached that some of the toxic symptoms produced by iproniazid may be due to an inhibitory effect on tyrosine oxidation.

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Isolation of Fully Deuterated Metabolites from *Scenedesmus obliquus* Grown in Deuterium Oxide

By M. I. BLAKE†, H. L. CRESPI, V. MOHAN, and J. J. KATZ

Successful mass culturing of *Scenedesmus obliquus* in 99.6 per cent deuterium oxide has proved a lucrative source of fully deuterated compounds. Whole cells are separated into three parts: (*a*) hot water extract, (*b*) crude pigments, (*c*) dry residue. The dry residue is hydrolyzed in acid. Five monosaccharides have been identified in the nonionic fraction of the hydrolysate. Deuterio-mannose and deuterio-glucose have been isolated in greater than gram amounts on a cellulose chromatographic column. Twelve amino acids have been detected in the ionic fraction. Deuterio-alanine, deuterio-glutamic acid, deuterio-aspartic acid, and deuterio-glycine have been separated in pure form by passage through a column of Dowex 50. The isolation, identification, and properties of these fully deuterated compounds are described.

THE SUCCESSFUL culturing of several species of algae in 99.6 per cent deuterium oxide has recently been reported (1, 2). The conditions for adaptation and autotrophic growth are described. The algae are grown in deuterium oxide with inorganic salts and carbon dioxide as the source of carbon. Under such conditions it is apparent that all compounds synthesized by the algae will have deuterium atoms where hydrogen atoms are ordinarily located. Since algae are rich in carbohydrates and proteins, a veritable storehouse of fully deuterated metabolites is now available. At present, many of these compounds cannot be conveniently synthesized by usual laboratory methods.

This paper describes the identification and isolation of a number of fully deuterated monosaccharides and amino acids from the carbohydrate and protein fractions of an algae hydrolysate. Routine procedures are in operation for obtaining these compounds from mass cultures of *Scenedesmus obliquus*. Additional fully deuterated metabolites will certainly be forthcoming as the procedures are refined.

EXPERIMENTAL

Mass Culturing of Algae.—The algae were grown in plastic rocking boxes of special design and contained 5 L. of nutrient solution. Carbon dioxide was fed as a mixture of 5% carbon dioxide and 95% nitrogen. The temperature was maintained at 26–28°. Continuous lighting was supplied by fluorescent lamps at an intensity of 1,000 foot-candles. All precautions were exercised to prevent exchange of the deuterium oxide with water of the atmosphere. The algae were agitated continuously by the rocking motion of the box. The growth medium had the composition (Gm./L.): $MgSO_4$ 0.50; NH_4NO_3, 2.00; Na_2HPO_4, 0.75; $KHCO_3$, 0.20; $Ca(NO_3)_2.4H_2O$, 0.03; NaCl, 0.02; $FeSO_4.7H_2O$, 0.045. The following trace elements were present (p. p. m.): boron, 0.75; manganese, 0.38; zinc, 0.08; copper, 0.03; molybdenum, 0.08. The algae were harvested twice a week. Complete details of the culturing procedure will be reported elsewhere.

The deuterium oxide used in this study had a purity of 99.6% and was distilled from alkaline permanganate in a Barnstead conductivity water apparatus.

General Fractionation Procedure.—The fractionation procedure is shown schematically in Fig. 1 and is described in detail in this section.

Harvests of algae culture were centrifuged in an International centrifuge at 2,000 r. p. m. for fifteen minutes. The supernatant liquid was discarded and the cells were washed once with fresh deuterium oxide. The algae were processed in convenient batches of 125–150 Gm. of wet packed cells. Each batch was slurried and added to about 1,500 ml. of boiling deuterium oxide and heating was continued for one and one-half minutes. As a result of this treatment the cell wall was ruptured and the cellular components were released. After cooling rapidly the solution was centrifuged. The supernatant liquid was lyophilized, producing about 9 Gm. of light brown powder which was stored in the freezer for further investigation. This material represents the deuterium oxide-soluble cellular constituents. The residue from the centrifugation was extracted twice with a methanol (2 parts) and petroleum ether (1 part) solvent mixture. The volume of solvent used was about ten times that of the packed cell volume. The crude pigments and lipids (12 Gm.) extracted by this solvent mixture were vacuum dried and stored in an evacuated glass bulb in the deep freeze for future study. The methanol-petroleum ether-insoluble fraction weighed 80 Gm. after vacuum drying. It is this material from which all deuterated metabolites reported in this study were isolated and represents essentially the cell walls of the algae. This material was then hydrolyzed by refluxing with 1 *N* deuterium chloride (10 ml. per Gm. of material) for twenty-four hours. The solution was filtered and the dried residue (15 Gm.) was

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Fig. 1.—Schematic diagram of general fractionation procedure. All isolated compounds contain deuterium atoms in nonexchangeable positions.

discarded. The hydrolysate was treated with silver carbonate to remove chloride. The solution was centrifuged and the residue discarded. The supernatant liquid was passed through a mixed bed ion exchange column (Amberlite MB-3), 4.0 cm. in diameter and 40 cm. in length. The column was washed with 1 L. of water. The eluate contained the nonionic fraction while the ionic material was retained by the column. The nonionic fraction was concentrated to a volume of 100 ml. on a steam bath, decolorized with charcoal, and evaporated to a thick syrup which weighed 34 Gm. The column was eluted with 500 ml. of 1 *N* hydrochloric acid and 300 ml. of water to remove the ionic fraction amounting to about 35 Gm. of material. This was examined for amino acid content as described in a subsequent section.

It should be noted that in the procedure water has been introduced. Therefore, all exchangeable deuterium atoms were obviously replaced by hydrogen atoms. It is a simple matter to reverse this process by dissolving the material in deuterium oxide and lyophilizing the solution. The amount of solvent calculated to effect a 99% exchange is employed. The process is repeated once. Nonexchangeable deuterium atoms will, of course, not be affected by water. Nevertheless, deuterium chloride was used for the hydrolysis step because of the drastic treatment involved. Once complete deuteration has been effected on a particular compound, the compound must be stored properly to avoid exchange in the presence of atmospheric moisture.

Chromatography of Nonionic Fraction.—A chromatographic column, 5.6 cm. in diameter, was packed with Whatman (standard grade) cellulose powder to a height of 60 cm. The column was washed with 2 L. of *n*-butanol:ethanol, 19:1; 1 L. of ethanol:water, 1:1; and 500 ml. of absolute methanol. The column was dried by suction for fifteen hours. The sugar mixture was poured on the column as a syrup (about 34 Gm.) forming a layer 2–3 cm. thick at the top of the column. The column was inverted and developed ascendingly with *n*-butanol:water, 19:1, for fifteen hours. The column was again inverted and the sugar mixture was eluted with a sequence of solvents indicated in the next section. Continuous operation was maintained by inverting a 1-L. volumetric flask, filled with solvent, at the top of the column. Fractions were collected with an automatic fraction collector, the turntable of which was adapted to hold 8-in. test tubes. The timing was adjusted so that 90-ml. fractions were collected. The fractions were evaporated to dryness in tared 125-ml. Erlenmeyer flasks. Each fraction was dissolved in sufficient 70% ethanol to yield a 1% solution. A 10-λ aliquot of each fraction and a known glucose solution were spotted on Whatman No. 1 chromatographic paper and developed descendingly for fifteen hours with *n*-butanol:acetic acid:water, 4:1:5, according to the method of Partridge (3). The papers were sprayed with benzidine reagent (4) which produced brown spots with the sugars. R_{ef} was calculated from the expression:

$$R_{ef} = \frac{R_f \text{ deuterated sugar}}{R_f \text{ glucose}} = \frac{\text{distance traversed by deuterated sugar}}{\text{distance traversed by glucose}}$$

The R_{ef} values for the monosaccharides identified in the sugar mixture are shown in Table I. In this study it was found that R_{ef} values were more consistent than R_f values and, in general, more useful.

Table I.—R_{ef} Values of Monosaccharides Identified in Nonionic Fraction

	R_{ef}(H)[a]	R_{ef}(D)[b]
Glucose	1.00	0.98
Mannose	1.26	1.22
Xylose	1.52	1.42
Ribose	1.87	1.74
Rhamnose	2.25	2.14

[a] Hydrogen amino acid. [b] Deuterated amino acid.

Isolation of Deuterio-mannose[1] and Deuterio-glucose.—Two solvent systems in sequence were used to remove the deuterated sugars from the column. The first solvent was *n*-butanol:water, 19:1. A total of 6 L. of eluate was collected in a large flask. Rhamnose, ribose, and xylose were identified in this fraction. Identification was based on chromatographic comparison with known sugars. Other components, not identified, were also present

[1] The prefix deuterio- is used here to indicate that nonexchangeable hydrogen atoms have been replaced with deuterium atoms.

TABLE II.—ANALYTICAL DATA FOR DEUTERATED METABOLITES

	Carbon, %		Hydrogen + Deuterium, %		Nitrogen, %	
	Calcd.	Found	Calcd.	Found	Calcd.	Found
Aspartic acid, $C_4H_4D_3NO_4$	35.29	35.53	7.40	7.44	10.29	10.01
Alanine, $C_3H_3D_4NO_2$	38.69	38.31	11.91	11.59	15.04	14.51
Glutamic acid, $C_5H_4D_5NO_4$	39.46	39.16	9.27	9.17	9.20	9.50
Glycine, $C_2H_3D_2NO_2$	31.16	30.92	9.15	8.97	18.17	18.04
Mannose, $C_6H_5D_7O_6$	38.49	37.94	10.23	10.13	...	...
Glucose, $C_6H_5D_7O_6$	38.49	38.40	10.23	10.19	...	...

TABLE III.—SPECIFIC ROTATION OF DEUTERATED METABOLITES

	Deuterated Compound, $[\alpha]_D^{25°}$	Hydrogen Compound, $[\alpha]_D^{25°}$
L-Alanine	+13.7	+14.3
L-Aspartic acid	+23.2	+25.4
L-Glutamic acid	+29.3	+31.5
D-Glucose	+48.5	+52.7
D-Mannose	+13.0	+14.6

in the eluate. The second solvent was *n*-butanol: ethanol, 19:1, saturated with water. At this point 90-ml. fractions were collected until a total of 5 L. of eluate was collected. These fractions were found to contain deuterio-mannose and deuterio-glucose. The column was then washed with 1 L. of ethanol: water, 1:1; 1 L. of methanol; and finally aspirated to dryness.

All fractions containing the same material, as indicated by paper chromatograms, were combined. The first fraction (6 L.) containing a number of components, was evaporated to dryness on a steam bath and set aside for characterization. It weighed 3.0 Gm. All fractions from the second solvent containing pure components were pooled, evaporated to dryness, dissolved in water, deionized, decolorized with charcoal, evaporated to dryness, and crystallized from either 90% ethanol (deuterio-glucose) or 97% ethanol (deuterio-mannose). Fractions containing both components were pooled and rerun on the cellulose column.

Analytical data and specific rotations for the isolated sugars are listed in Tables II and III.

Chromatography of Ionic Fraction.—The amino acids present in the ionic fraction were identified by two-dimensional chromatography. A 10-λ aliquot of an aqueous solution of the ionic fraction was spotted in one corner of a sheet of Whatman No. 1 chromatographic paper ($18^1/_4 \times 22^1/_2$ in.). The paper was placed in a Chromatocab and developed descendingly with *n*-butanol:acetic acid:water, 4:1:5, for seventeen hours; then dried at room temperature, rotated 90 degrees, and developed in the second direction with 78% phenol in water for twenty-four hours. After drying at room temperature, the paper was sprayed with ninhydrin reagent (2.0 Gm. ninhydrin, 8 ml. of s-collidine, 500 ml. of 95% ethanol), and dried in special chromatographic oven at 70° for five minutes to effect the color reaction. A typical chromatogram is illustrated in Fig. 2. Identification of the amino acids was based on comparison of R_f values. The R_f values for the hydrogen amino acids were determined in each solvent system and are reported in Table IV. Values for the deuterated amino acids

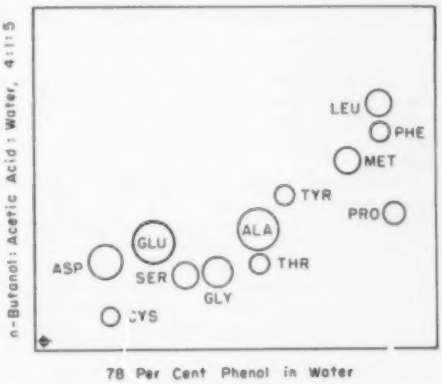

Fig. 2.—Two-dimensional chromatogram of ionic fraction.

TABLE IV.—R_f VALUES OF AMINO ACIDS IDENTIFIED IN IONIC FRACTION

Amino Acid	Phenol-Water		*n*-Butanol-Acetic Acid-Water	
	R_f(H)[a]	R_f(D)[b]	R_f(H)	R_f(D)
Cystine	0.17	0.17	0.06	0.06
Aspartic acid	0.19	0.17	0.23	0.21
Glutamic acid	0.29	0.26	0.23	0.21
Serine	0.40	0.36	0.18	0.16
Glycine	0.43	0.40	0.19	0.17
Threonine	0.56	0.53	0.23	0.22
Alanine	0.61	0.58	0.33	0.30
Tyrosine	0.66	0.63	0.42	0.41
Methionine	0.79	0.76	0.51	0.49
Leucine	0.86	0.82	0.64	0.62
Phenylalanine	0.86	0.80	0.59	0.56
Proline	0.89	0.86	0.36	0.32

[a] Hydrogen amino acid. [b] Deuterated amino acid.

were determined from the two-dimensional chromatogram and are also reported in Table IV. The ionic fraction was cochromatogramed with the hydrogen amino acids. There was a darkening in the intensity of the spots but no observable separation was noted. A synthetic mixture was prepared containing those amino acids (but not deuterated) identified in the ionic fraction. It produced a chromatogram identical to that of the ionic fraction.

Ion exchange chromatography was used to fractionate the ionic fraction. A modification of the procedure described by Carsten (5) and Stein and Moore (6) was employed in this study.

Two hundred grams of Dowex 50-X8 cation exchange resin (200–400 mesh) was washed repeatedly with 10% solution of sodium carbonate by decanta-

tion. The resin was then washed several times with distilled water and finally with 10% hydrochloric acid to convert the resin to the hydrogen form. A column was prepared by pouring the resin, as a slurry, into a long chromatographic tube having an inner diameter of 2.5 cm. The length of the packed column was 100 cm. Distilled water was passed through the column until the pH of the eluate was 5.0. Continuous operation of the column was maintained by means of a reservoir connected to the top of the column by suitable length of Tygon tubing. The reservoir was a 500-ml. round-bottom flask with a small tapered opening at the base to which the tubing was joined. Rate of flow of eluate was controlled by adjusting the height of the reservoir. Eluate was collected in 4-ml. test tubes arranged on the turntable of an automatic fraction collector. In this study the flow rate was maintained at 5.0 ml. per hour (actually 2.5 ml. per tube per thirty minutes).

The ionic fraction of the algae hydrolysate was a thick syrup having a light amber color. The extract was layered on the surface of the resin with the aid of a pipet. The reservoir, containing distilled water, was fixed to the column. A total of 250 ml. of eluate was collected in a beaker. At this point fractions were not collected. Nonionic components, chiefly carbohydrates, were removed by the water. The eluant was then changed to 0.1 *M* hydrochloric acid and fractions were collected with the automatic fraction collector. The molarity of the acid was progressively increased to 4 *M*. A total of 2,000 ml. of eluate was collected.

Each fraction of eluate was tested qualitatively for the presence of amino acids. The fractions were spotted on a line parallel with the long dimension of Whatman No. 1 chromatographic paper. The paper was developed descendingly with 78% phenol in water. The amino acid composition of each fraction was determined from the R_f values.

Isolation of Deuterated Amino Acids.—All fractions containing the same amino acid were combined. Where overlapping occurred, the fractions were rechromatogramed. The combined fractions were evaporated by dryness at room temperature. The residue was dissolved in 10 ml. of water; the solution was decolorized with charcoal, heated to boiling, and filtered. The pH of the solution was adjusted to the isoelectric point of the amino acid. Fifty milliliters of 95% ethanol was added and the solution was placed in the refrigerator until crystallization occurred. The amino acid was filtered off and washed with ether. The identity of the amino acid was established by chromatograming 5λ of a 1% solution of the isolated acid, 5λ of a solution of known acid, and 5λ of a mixed solution of both known and unknown material. The R_f values were then compared. Analytical data and specific rotation of the deuterated amino acids obtained in pure form are listed in Tables II and III.

RESULTS AND DISCUSSION

Algae are composed chiefly of protein, lipid, and carbohydrate, but the relative concentration of each may vary considerably (7), depending on environmental conditions. The abundance of a particular component may be altered by the proper selection of growth conditions. Therefore, the weights of deuterated metabolites indicated in Fig. 1 are significant only for *S. obliquus* grown under the conditions described here.

In this investigation only the carbohydrate and protein fractions of the cell wall were studied as sources of fully deuterated metabolites. After hydrolysis, satisfactory, but not complete, separation of the sugars (nonionic fraction) from the amino acids (ionic fraction) was effected by passage through a column of Amberlite MB-3. Nonionic contaminants in the ionic fraction were conveniently removed from the Dowex 50 column by washing with water and were subsequently fractionated in the usual manner on the cellulose column. Five monosaccharides have been identified in the nonionic fraction by paper chromatographic analysis. R_{ef} values are listed in Table I and are relative to ordinary glucose (not deuterated) as a standard. It is apparent that the deuterated derivatives move at a slightly slower rate than the corresponding hydrogen compounds, probably because of lower solubility in the developing solvent.

The amino acid composition of the ionic fraction, determined by two-dimensional chromatography, is shown in Fig. 2. The approximate relative abundance is indicated by the size of the spot. Twelve amino acids have been identified. R_f values for two solvent systems are shown in Table IV. Values for the deuterated compounds are consistently lower than the hydrogen compounds. It is probable that other amino acids are present in the algae, but are not present in the cell wall, or, if present, the abundance is too small to be detected by the techniques employed here. Attempts to resolve mixtures of the deuterated and hydrogen forms of the amino acids by paper chromatography were unsuccessful. However, it is noteworthy that Strain, *et al.* (8), have separated β-carotene from deuterio-β-carotene (isolated from the lipid fraction as indicated in Fig. 1) into distinct zones on a magnesium oxide column.

Deuterio-mannose and deuterio-glucose have been isolated in greater than gram amounts from the sugar fraction, while deuterio-aspartic acid, deuterio-glycine, deuterio-alanine, and deuterio-glutamic acid have been obtained in pure form from the amino acid fraction. The analytical data for these compounds are presented in Table II. The calculated elemental per cent is based on the empirical formula shown with the chemical name. The relative number of deuterium atoms to hydrogen atoms, as indicated by the empirical formula was confirmed by analysis of the water of combustion for deuterium. The method of analysis has been described earlier (2). The hydrogen + deuterium per cent was determined by the usual combustion technique using the proper conversion factor based on the number of hydrogen and deuterium atoms in the empirical formula.

Optical properties of the isolated compounds are reported in Table III. The configuration, direction, and extent of rotation are indicated for both the deuterated and hydrogen compounds. The values for deuterio-mannose and deuterio-glucose were taken after mutarotation was complete. Initial readings indicated that both were of the alpha series prior to mutarotation. Differences in specific rota-

tion may be attributed to the effect of deuterium atoms on the asymmetry of the molecule.

Infrared spectra of these compounds are currently under investigation and a detailed analysis will be reported in a subsequent publication.

The availability of fully deuterated carbohydrates and amino acids should lead to many interesting kinetic isotope studies and biological investigations. A number of such studies are currently in progress in this laboratory. Further work is also directed to the isolation of other metabolites identified in the algae.

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Viscosity-Stability of Aqueous Solutions of Certain Hydrophilic Polymers

By GERHARD LEVY

The viscosity-stability of sodium carboxymethylcellulose and sodium alginate, in aqueous solution, has been investigated. The "viscosity half-life" of sodium carboxymethylcellulose decreases with increasing average molecular weight and increases with concentration. The "viscosity half-life" of sodium alginate also decreases with increasing molecular weight, but appears to be independent of concentration in the concentration range studied. The stereochemical features of these polymers, which may serve to explain the concentration effect, are discussed. The viscosity-stability of sodium alginate increases with increasing calcium content. Methods for a meaningful comparative evaluation of the viscosity-stability characteristics of hydrophilic polymers are discussed.

HYDROPHILIC POLYMERS of high molecular weight are used in pharmacy primarily because of the pronounced viscosity which they impart to aqueous systems. This property makes them useful suspending and stabilizing agents. The stability of these compounds is as important as that of the therapeutic agents with which they are usually combined. Degradation and the resulting reduction in the viscosity of polymer solutions may cause accelerated sedimentation of the formerly suspended therapeutic agents and could result in the accidental ingestion of overdoses of potent medicinals. Furthermore, the flow properties and elegance of drug products may be sufficiently impaired to reduce their patient-acceptance.

Despite the general interest in the stability of medicinals, relatively few studies of the viscosity-stability of pharmaceutical viscosity-enhancing agents have appeared in the literature. Yet, these compounds generally present marked stability problems by reason of their polymeric nature, since they are subject to chain scission which causes a decrease in the viscosity of their solutions.

The degradation of high polymers has been studied extensively (1–3) but the kinetics of the degradation processes have been expressed primarily in terms of the number of bonds broken or the decrease in average molecular weight as a function of time. In the case of polysaccharides, which constitute the largest group of pharmaceutically important viscosity-enhancing agents, the hydrolytic degradation is essentially a random process in which all interunit bonds are equally susceptible to attack (4, 5). An exception are the terminal bonds, which are somewhat more reactive. Their effect can usually be neglected, since their proportion in high molecular weight polymers is exceedingly small. There have also been reports of "weak links" present in small number and spaced evenly along the length of certain cellulosic compounds (3, 6). These weak links are said to be more readily ruptured than the normal β-glycosidic linkages and may ac-

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count for a relatively higher degradation rate in the initial phases of a depolymerization process.

In view of the relationship of molecular weight to solution viscosity, the change in the molecular weight of polymers undergoing degradation is of special interest. If, for example, the bond scission in a certain high polymer occurs only at the terminal ends (i.e., a "peeling off" process), the molecular weight changes little and the viscosity of the solution is relatively unaffected. If, on the other hand, the cleavage involves a central bond in the polymer molecule, the molecular weight is halved and the viscosity may be appreciably decreased. Thus it is not only important to consider the rate of bond cleavage but also the site at which the bonds are broken. It has been calculated (7) that, in the case of entirely random cleavage, the rupture of about 2.8 glycosidic bonds in a dissolved linear macromolecule will reduce the average chain length by one-half.

Since the chance of a chain break becomes greater as the degree of polymerization (D. P.) increases, the time required for the reduction of the average molecular weight to one-half its initial value should decrease with increasing degree of polymerization. Because the viscosity of polymer solutions is proportional to the molecular weight of the polymer, it follows that the viscosity-stability of aqueous solutions of linear polymers undergoing random bond scission should vary inversely with molecular weight. Unfortunately, very little consideration has been given in pharmacy to the effect of molecular weight upon the stability of hydrophilic polymers, despite the known variation in the D. P. of these compounds either by nature or by design through fractionation.

It has been shown (8, 9) that the rate of decrease of the number average molecular weight of randomly degrading polymers is proportional to the square of their molecular weight at a given time. It is impossible, however, to translate kinetic relationships which are based either upon the rate of decrease of the average molecular weight or the rate of bond scission, into viscosity changes. For one, the viscosity of non-Newtonian systems is not a constant but a variable affected by shear rate. Staudinger proposed an empirical viscosity-molecular weight relationship which is now usually expressed as $[\eta] = K_m M^a$ where K and a are constants, $[\eta]$ is the intrinsic viscosity or limiting viscosity number, and M is the weight-average molecular weight (10). This relationship refers however to the limiting value of the reduced viscosity at zero concentration in order to minimize concentration effects. No adequate relationship between molecular weight, concentration, and viscosity of non-Newtonian solutions of polymeric compounds has as yet been evolved.

An additional complicating factor is the heterogeneity of these substances. The average molecular weight represents only a limited characterization of a high polymer; the distribution of molecular sizes about the average (the chain length distribution or polydispersity) must also be considered. Even if it were possible to obtain a relatively uniform material by efficient fractionation, the chain length distribution broadens markedly as depolymerization proceeds, attaining a maximum width when the average molecular size is reduced to approximately one-half (11). Subsequently, the polymeric compound becomes more homogeneous again as monomeric constituents accumulate. For all these reasons, the relation of the viscosity-stability of solutions of hydrophilic polymers to their average molecular weight must be studied experimentally.

This report deals with a study of the viscosity-stability of aqueous dispersions of two pharmaceutically important hydrophilic polymers, sodium alginate and sodium carboxymethylcellulose, as a function of their average molecular weight. The experimental design takes into consideration certain special properties of these polymers: they are subject to chain degradation by shearing forces, especially at higher concentrations and molecular weights (12, 13). It has also been reported that the rheological characteristics of solutions of certain types of sodium carboxymethylcellulose may be affected by the shear intensity applied in their preparation (14). Accordingly, solutions of various concentrations of each polymer have been prepared by dilution of the most concentrated solution. In this manner, the extent of shear degradation is identical at each concentration.

While the rate of degradation in terms of the proportion of bond lyses per unit time is independent of concentration in dilute solution (15), aggregation of polymer molecules at higher concentrations might affect their degradation rate. Evidence for strong interaction between sodium carboxymethylcellulose molecules has been reported by Allgen and Roswall (16). For this reason, viscosity-stability determinations have been carried out at various concentrations chosen so that each concentration yields solutions with an apparent viscosity approximately equal to that of solutions prepared from the other molecular weight types of each polymer.

Both polymeric compounds investigated are anionic in nature, possessing numerous carboxyl groups. The presence of polyvalent cations in

such systems may result in inter- and intramolecular bridges which may sterically, and by their effect upon the degree of hydration, affect the rate of depolymerization. By reason of the processing methods used in the preparation of sodium alginate, considerable quantities of residual calcium may be present in pharmaceutical grades of sodium alginate (17). Accordingly, the effect of various concentrations of calcium on the viscosity-stability of sodium alginate solutions has also been investigated.

EXPERIMENTAL

Materials.—Three types of extra refined sodium alginate, XR-A, XR-B, and XR-C, varying only in their degree of polymerization (18), have been used for the preparation of the sodium alginate solutions. The average molecular weight as well as the calcium and moisture content of these materials is shown in Table I.

TABLE I.—SODIUM ALGINATE SPECIFICATIONS

Type	Av. Molecular Weight[a]	Calcium Content,[b] %	Loss on Drying,[c] %
XR-A	46,000	0.16	12.2
XR-B	109,000	0.21	13.2
XR-C	113,000	0.15	11.9

[a] Determined by intrinsic viscosity measurements, Donnan, F. G., and Rose, R. C., *Can. J. Research*, **28B**, 105(1950).
[b] Per cent of calcium based on undried sodium alginate. Analyses by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.
[c] Procedure of National Formulary, 10th ed., p. 690.

The following types of sodium carboxymethylcellulose have been used: CMC-7HP, CMC-7MP, and CMC-7LP. The average molecular weights of these compounds are approximately 250,000, 75,000, and 40,000, respectively (19). They represent a premium grade material with a degree of substitution from 0.65 to 0.85 (20).

Preparation of Solutions.—All solutions were prepared with distilled water containing 0.1% methylparaben and 0.025% propylparaben to prevent microbial growth. The highest desired concentration of each material was prepared and placed on an automatic shaker for one hour. The solution was then permitted to stand for twenty-four hours, shaken well, and the more dilute solutions were prepared from it by addition of the appropriate quantities of paraben water.

All solutions were then again placed on an automatic shaker for one hour. The next day the solutions were poured into eight-ounce round glass jars which were tightly capped. The initial viscosity of the solutions was determined at this time and the jars were placed in the oven.

The sodium alginate solutions were prepared in portions of 300 ml. in a Waring Blendor controlled by means of a Powerstat variable transformer. The gum was sprinkled on the paraben water at room temperature and the dispersion was agitated for exactly five minutes at a Powerstat variable transformer setting of 60. The various 300-ml. portions of solution thus prepared were then consolidated.

For the investigation of the effect of calcium upon the viscosity-stability of sodium alginate solutions, a 3% solution of sodium alginate XR-C was prepared in the manner described above. The appropriate amount of calcium chloride was dissolved in paraben water and the two solutions were mixed to yield 2% sodium alginate solutions containing 0.5, 1.0, and 1.5% of added calcium based on the weight of sodium alginate. Controls containing no added calcium were also prepared. In addition, every solution contained an amount of sodium chloride sufficient to maintain the combined ionic strength of the added calcium chloride and sodium chloride equivalent to 0.5% sodium chloride.

The sodium carboxymethylcellulose solutions were prepared by dispersion of the gum with a high-shear automatic mixer in 300-ml. portions of paraben water preheated to 80°. After two minutes of such treatment, and after all portions of the solution had been consolidated, they were mixed with a Brookfield counter-rotating mixer set at 50 for one-half hour.

Viscosity Determinations.—The solutions under study exhibited non-Newtonian flow characteristics and required a rigidly standardized method for the determination of their viscosity. In order to facilitate a comparative evaluation, the apparent viscosity was determined at a constant shear rate. For this purpose, the Brookfield Synchro-Lectric viscometer, model LVT, was utilized. The apparent viscosities were determined at 30 ± 0.1°, with a No. 4 spindle, at 12 r.p.m. and after ten minutes of spindle rotation. The apparent viscosity values mentioned in this paper would be equivalent to centipoise units in the case of Newtonian liquids.

Stability Studies.—The various solutions, contained in tightly capped eight-ounce glass containers, were stored at 49 ± 1° in a mechanical convection oven. Under these conditions, the solutions were protected from any natural or artificial light. All experiments were carried out in quadruplet and the reported data are the averages of four determinations. At appropriate intervals, 30-ml. samples were rapidly removed from each jar for viscosity determinations. These samples were then discarded. None of the stored samples exhibited any gross microbial contamination during the stability test runs.

RESULTS AND DISCUSSION

Figures 1, 2, and 3 show the change in the apparent viscosity of solutions of the high, intermediate, and low molecular weight types of sodium alginate. The viscosity-degradation curves exhibit first-order characteristics in the case of the high and intermediate molecular weight types of sodium alginate, but the low molecular weight sodium alginate solutions deviate somewhat from such behavior. The effect of concentration upon the stability appears to be negligible in the concentration range studied. It can be seen, however, that the viscosity-stability of the solutions is markedly affected by the average molecular weight. The solutions prepared from low molecular weight sodium alginate exhibit a viscosity half-life that is four to five times greater than that of the high molecular weight sodium alginate solutions.

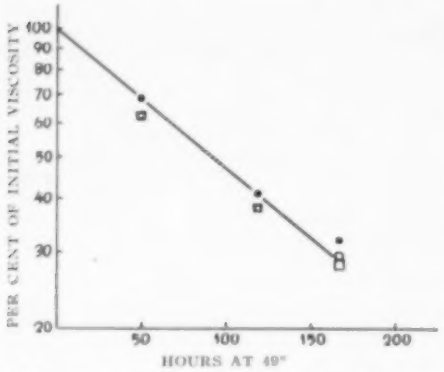

Fig. 1.—The viscosity-stability of sodium alginate XR-C (mol. wt. ~ 113,000). ● 2.8%, ○ 2.5%, □ 2.0%.

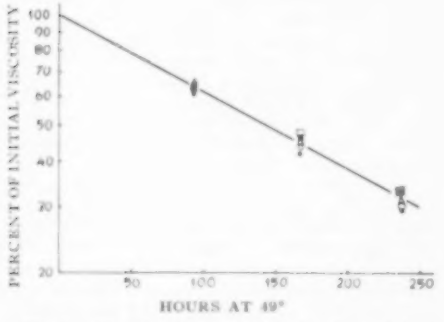

Fig. 2.—The viscosity-stability of sodium alginate XR-B (mol. wt. ~109,000). ● 2.3%, △ 2.5%, ○ 2.7%, × 2.9%, □ 3.1%.

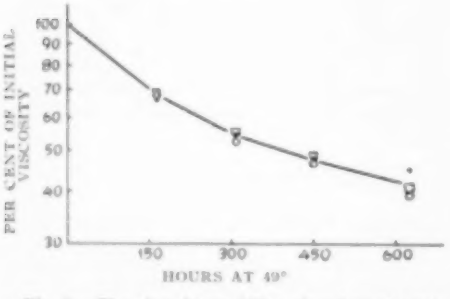

Fig. 3.—The viscosity-stability of sodium alginate XR-A (mol. wt. ~46,000). ● 6.5%, ○ 6.0%, □ 5.0%.

The difference in the stabilities of the intermediate and high molecular weight sodium alginate appears to be greater than would be expected on the basis of their average molecular weight. The latter was estimated by means of intrinsic viscosity measurements. These measurements yield weight-average molecular weights in which the heavy molecules are favored (21, 22). Fractionation of the high and intermediate molecular weight sodium alginates indicates that the latter contains a greater proportion of low molecular weight molecules. This would account for the extent to which the viscosity-stabilities of the two materials vary.

The apparent viscosity *versus* time curves of sodium alginate solutions containing various concentrations of calcium exhibited essentially first-order characteristics. Figure 4 represents the change in viscosity half-life as a function of total calcium. Calcium appears to have a marked effect upon the viscosity-stability; as little as 1.5% total calcium (based on the weight of sodium alginate) doubles the viscosity half-life. At that concentration, about 15% of the carboxyl groups in the alginate molecules would be cross-linked.

The concentrations and initial apparent viscosities of the various sodium alginate solutions are listed in Table II.

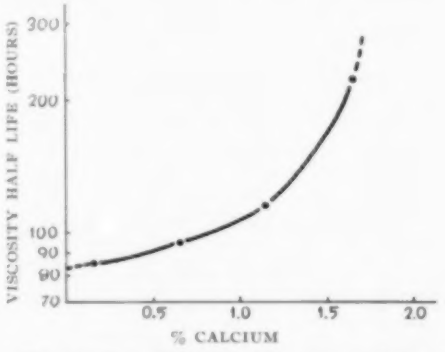

Fig. 4.—The effect of small concentrations of calcium on the viscosity-stability of 2.0% solutions of sodium alginate XR-C. (Percentage of calcium is based on sodium alginate powder).

Table II.—Initial Viscosity of Sodium Alginate Solutions

Type	Concentration, %	Initial Apparent Viscosity[a]
XR-A	7.0	19,800
XR-A	6.5	15,300
XR-A	5.0	6,000
XR-B	3.1	16,500
XR-B	2.9	12,800
XR-B	2.7	10,600
XR-B	2.5	8,000
XR-B	2.3	5,900
XR-C	2.8	18,400
XR-C	2.5	13,500
XR-C	2.0	6,300
XR-C (NaCl control)	2.0	7,600
XR-C (0.5% added Ca)	2.0	7,300
XR-C (1.0% added Ca)	2.0	8,400
XR-C (1.5% added Ca)	2.0	47,000

[a] Average of four samples.

Figures 5, 6, and 7 represent the decrease in the apparent viscosity of sodium carboxymethylcellulose solutions as a function of time. Of interest is the initial rise in the apparent viscosity of the low and intermediate molecular weight sodium carboxy-

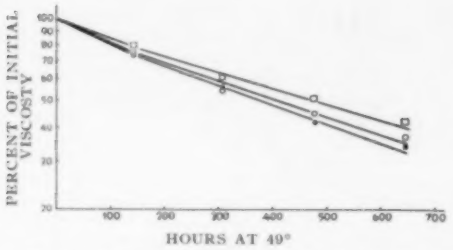

Fig. 5.—The viscosity-stability of sodium carboxymethylcellulose solutions (mol. wt. ~250,000). ● 1.3%, ○ 1.6%, □ 1.9%.

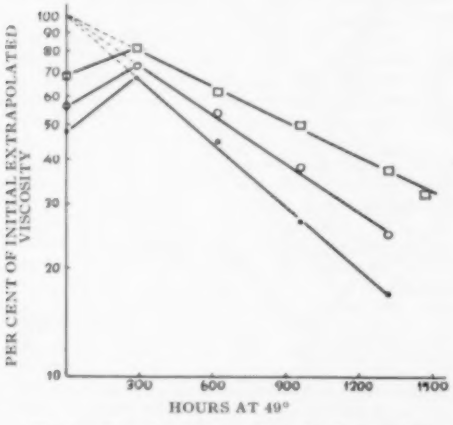

Fig. 6.—The viscosity-stability of sodium carboxymethylcellulose solutions (mol. wt. ~75,000). ● 3.25%, ○ 3.50%, □ 3.75%.

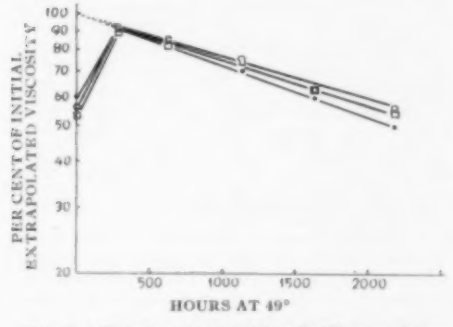

Fig. 7.—The viscosity-stability of sodium carboxymethylcellulose solutions (mol. wt. ~40,000). ● 6.0%, ○ 6.5%, □ 7.0%.

methylcellulose solutions as well as the marked dependence of viscosity-stability upon concentration. The initial rise in the apparent viscosity is more pronounced in the case of the low than in the intermediate molecular weight solutions and is not found in the solutions of high molecular weight gum. This viscosity build-up might be due to slow hydration or to entanglement of the polymer chains. The absence of the initial viscosity build-up in solutions of the high molecular weight polymer may be due to the more rapid chain entanglement of the larger molecules or to a rapid initial degradation rate which could mask the viscosity build-up. These interpretations are entirely speculative and the observed phenomenon requires further elucidation.

The pronounced effect of concentration upon the viscosity-stability of sodium carboxymethylcellulose solutions might result from the strong interaction of the molecules of this polymer (16). Such interaction, leading to less polar aggregates, could reduce the degree of hydration of the compound and make the glycosidic linkages less accessible to hydrolytic cleavage. The extent of intermolecular association, and for that reason the resistance to depolymerization, can be expected to increase with concentration. Russian workers who studied the hydrolysis rate of methylcellulose in sulfuric acid solution have also found that an increase in polymer concentration resulted in a decrease in hydrolysis rate (23).

It appears unlikely that the greater stability exhibited at higher concentrations represents a reduction in oxidative degradation of the gum due to the slower diffusion of oxygen at higher viscosities. The solutions were neutral in pH, protected from light, and kept in tightly closed containers. Such conditions favor hydrolytic rather than oxidative degradation (24).

When comparing the viscosity-stability of solutions of sodium carboxymethylcellulose of various average molecular weights, the effects of concentration must be considered. It serves no useful purpose to compare the stabilities of solutions of equal concentration; it is more meaningful to compare solutions of equal viscosity, since viscosity rather than polymer concentration is usually the invariable in the preparation of pharmaceuticals.

Figure 8 represents a plot of viscosity half-life *versus* the initial apparent viscosity of the three

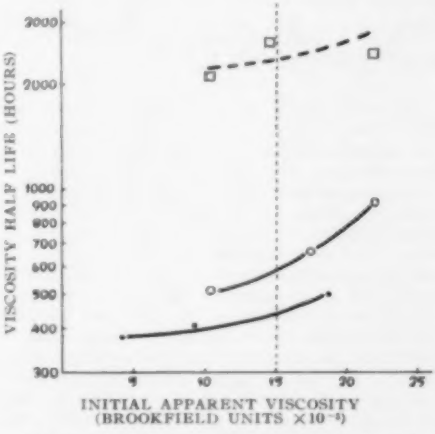

Fig. 8.—The viscosity half-life ($V_{1/2}$) of sodium carboxymethylcellulose solutions at 49° as a function of the initial apparent viscosity. The vertical line intersects the $V_{1/2}$'s at an apparent viscosity of 15,000. □ mol. wt. ~40,000, $V_{1/2}$ 2,400 hours; ○ mol. wt. ~75,000, $V_{1/2}$ 580 hours; ● mol. wt. ~250,000, $V_{1/2}$ 440 hours.

types of sodium carboxymethylcellulose studied. The viscosity half-life is based upon the extrapolated degradation curves in the case of the low and medium molecular weight polymers. The vertical line in Fig. 8 serves to compare the viscosity half-lives of the three polymer types at a given apparent viscosity. The lower molecular weight type is more than five times as stable as the high molecular weight type under the conditions of the test. Comparison of solutions of equal concentration rather thanequal viscosity would give a distorted and misleading picture of their relative stabilities to the pharmaceutical formulator. It is of interest that the effect of concentration upon viscosity-stability is not limited to the unbranched, linear methylcellulose and sodium carboxymethylcellulose molecules. Figure 9 shows a similar effect for guar, which has single-membered galactose branches attached to every other mannose unit of the chain (25). The viscosity-stability of the linear polymer sodium alginate, on the other hand, appears to be relatively independent of concentration.

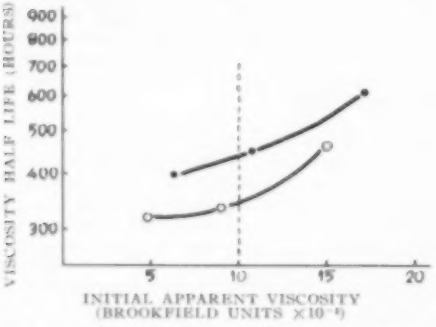

Fig. 9.—The viscosity half-life of guar solutions at 49° as a function of initial apparent viscosity. ● Jaguar GR-80 1.3, 1.5, and 1.7%. ○ Jaguar A-20-A 0.9, 1.1, and 1.3%. $V_{1/2}$ at an apparent viscosity of 10,000 (shown by vertical line) is 440 and 350 hours, respectively.

A stereochemical analysis of the structural features of these polymers provides greater insight into the concentration-stability relationships. Since the intermolecular association of polysaccharides is largely mediated through hydrogen bonding of their hydroxyl groups, the accessibility of these groups would influence the degree of intermolecular association. The conformations of the monomers of sodium carboxymethylcellulose and sodium alginate, as well as of the galactose branches on the guar chain, are shown in Fig. 10. The two to three hydroxyl groups (depending on the degree of substitution) on the methylcellulose and carboxymethylcellulose monomer units tend to occupy the equatorial positions. The same is true for the hydroxyl groups on the galactose units of guar. On the other hand, only one of the hydroxyl groups on the alginate monomer is in equatorial position, the other is axial. The equatorial hydroxyl groups are more readily accessible for intermolecular hydrogen bonding than those in axial position (26). For this reason, one may expect greater interaction among methylcellulose, carboxymethylcellulose and guar molecules than among alginate molecules. Since intermolecular hydrogen bonding increases with concentration, and since molecular association tends to decrease hydration and shield the glycosidic linkages from hydrolysis, the increased stability of guar, carboxymethylcellulose, and methylcellulose at higher concentrations can be explained on the basis of the equatorial position of all their hydroxyl groups. The relative lack of concentration effects upon the stability of sodium alginate, on the other hand, appears to be due to the *cis* position of the hydroxyl groups, and the hydration and repelling effect of the carboxyl groups.

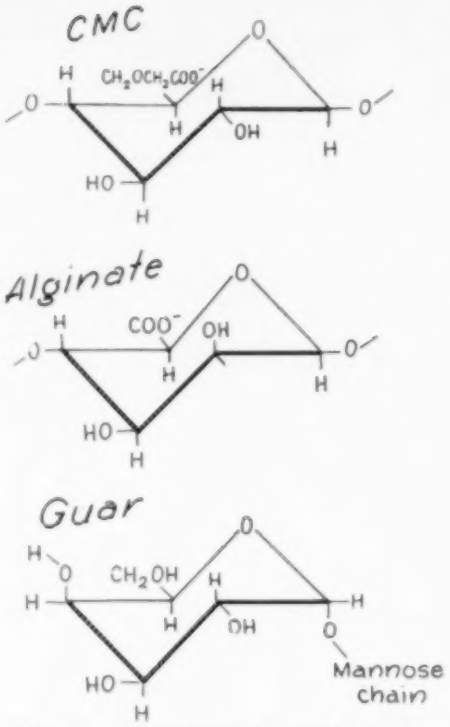

Fig. 10.—Conformation of typical monomer units of sodium carboxymethylcellulose, sodium alginate, and the galactose branches on guar gum.

In conclusion, general considerations with respect to the viscosity-stability of hydrophilic polymer solutions may be outlined. The viscosity-stability of solutions of linear polymers subject to random degradation generally varies inversely with the molecular weight of the polymer. The weight average molecular weight estimated by intrinsic viscosity determination, or even the apparent viscosity of a solution of standard concentration, provides a useful estimate of the relative stability of different types of a given polymeric compound. Additional consideration may have to be given to the molecular weight distribution within the compound. This becomes important in cases where different viscosity-types of a gum are prepared by the blending of high and low molecular weight materials, a common commercial practice.

Several factors specific to individual hydrophilic polymers must also be considered. For example, the presence of significant quantities of residual calcium in sodium alginate might give a misleading impression of high molecular weight due to the high viscosity of solutions prepared from this material. The presence of calcium in such solutions is easily determined by the addition of chelating agents and the resulting viscosity drop, or on the basis of the characteristic freeze-thaw behavior of calcium-containing sodium alginate solutions (17).

Sodium carboxymethylcellulose is not only available in different average molecular weight types, but also in different degrees of substitution (D. S.). The low D. S. products are more stable to hydrolytic degradation than those with a high D. S. (24).

The character of the viscosity-time plot is another factor to be considered. Random degradation of linear polymers is generally considered to be first-order with respect to the number of unbroken bonds in the polymer (3, 27). This is reflected by the viscosity *vs.* time curves obtained with the hydrophilic polymers studied. Tragacanth gum, on the other hand, exhibits apparent zero-order degradation in a plot of plateau viscosity against time (28). This may be due to a "depot" of undissolved gum which serves to maintain a constant concentration of tragacanth in solution.

Certain polymer characteristics sometimes tend to obscure the character of the degradation curve, for example, the initial viscosity increase of certain sodium carboxymethylcellulose solutions and the very interesting effect of method of preparation on the viscosity and stability of tragacanth solutions (28, 29).

Hydrophilic polymers subjected to excessive shear and high temperatures during manufacture of pharmaceutical products may be partially depolymerized. This results not only in lower initial viscosities but also in a reduction in the magnitude of subsequent viscosity changes. Thus we can explain, in retrospect, the observation by Gabel (30) that "neutralized mucilages of tragacanth made with the aid of heat showed a stable viscosity, though decidedly less viscous than mucilages made without neutralization and heat." Also, that low viscosity types of natural gums are generally more stable than the high viscosity types.

The comparison of viscosity half-lives of iso-viscous rather than iso-concentrated solutions affords a more realistic means of evaluating the stabilities of hydrophilic polymers differing in average molecular weight. Where stability considerations are paramount and higher solid content is no objection, a gum of low molecular weight would generally be the preferred choice. Sometimes, however, flow characteristics of gums change with molecular weight. For that reason, the effect of D. P. on the non-Newtonian index (31) of the solutions must also be taken into consideration. Finally, the effect of electrolytes, nonaqueous solvents, pH, and other factors upon the viscosity-stability of a gum may affect the ultimate choice of the polymeric compound to be used in a pharmaceutical product.

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Actions of Estrogens on Blood and Bone Marrow

By LAURETTA E. FOX

Chlorotrianisene and estradiol produced neutrophilia in strain 101 mice. Estrogens were without action in tissue culture, but bone marrow of mice pretreated with estrogens showed evidence of faster differentiation of cells of the granulocytic series than bone marrow from control mice.

MANKOWSKI (1) reported that Swiss white mice receiving estradiol developed neutrophilia. Fox, *et al.* (2), reported variable red and white cell counts of Swiss white mice administered estrogens. Girod (3–5) reported that leukocyte responses are variable in rats, but more stable in rabbits and monkeys.

The purpose of this research was to determine whether an inbred strain of mice might produce uniform erythrocytic and leukocytic responses to estradiol and chlorotrianisene.

METHODS

Strain 101 mice, nine to ten weeks old, were maintained on Purina chow and water *ad libitum* in a room maintained at 72 ± 2° F. for three weeks prior to these experiments. The sexes were housed separately. The mice of each sex were randomly divided into six groups as indicated in Tables I and II. Mice were injected with estradiol, one dose of 0.05 mg. or chlorotrianisene, one dose of 0.5 mg. in sesame oil, on the fourteenth day of the experiment (after blood was drawn for counts and smears).

Each time before blood was drawn for counting the groups were placed in glass jars under an electric light. When the temperature of a thermometer, placed at the level of the mice, reached and was maintained at 37° for fifteen minutes the blood samples were taken, diluted, and counted according to standard techniques (6). Blood smears were stained with Giemsa-Wright stain.

The male mice were killed by exsanguination and the femur bone marrow removed aseptically. An aliquot was taken in a leukocyte diluting pipet. It was diluted and counted like a blood sample for a total leukocyte count (7).

The bone marrow was cultured in spinner flasks in a hood maintained at 37.0 ± 1.0°. The tissue culture medium consisted of: T. C. medium 199, 68.97%; T. C. medium 109, 17.24%; horse serum, 11.49%; and bovine embryo extract, 2.3%.

The flasks were supplied constantly with a stream of 5% carbon dioxide in oxygen flowing at a rate of 0.15 L./min. The medium was agitated with a magnetic stirrer. Culture medium dropped by gravity from a reservoir at a rate of 1.0 ml./hr. into the spinner flask. The constant addition of fluid prevented loss of cells in the meniscus of the flask. Changes in volumes in the flasks were considered in the calculation recorded in Table III.

Three cultures were used in each group. Sufficient estradiol was added to three cultures of group *1A* to make a concentration of 0.5 γ per ml. of medium and sufficient chlorotrianisene to three cultures of group *1B* to make a concentration of 5.0 γ per milliliter.

Aliquots of these cultures were withdrawn four, eight, twelve, and thirty-six hours after culture started. The number of nucleated cells was counted. Smears were prepared and stained with Giemsa-Wright stain. An adaptation of the method of Berman and Powsner (8) was used to score the maturation rate of the cells on the smears. Standard methods were used to determine the number of granulocytes per hundred nucleated cells.

Femoral bone marrow of three male mice pretreated with chlorotrianisene, group II, and three pretreated with estradiol, group III, were cultured and studied by the methods cited above.

RESULTS AND DISCUSSION

Strain 101 mice gave more uniform red and white blood cell counts than Swiss white mice (2). There was less variation in the red blood cell counts of male than female mice (Table I).

Neither the estradiol nor the chlorotrianisene, under the experimental conditions used, produced significant changes in red or white (Table II) blood cell counts, confirming the earlier studies of Mazer, Meranze, and Israel (9) on estradiol. Counts on days twenty-one and twenty-eight showed no significant changes from the counts recorded in Tables I and II.

The average differential count for each group was recorded in Table IV. One day after injection of estradiol and three days after injection of chlorotrianisene the number of neutrophils increased. Simultaneously the per cent of lymphocytes tended to decrease.

Neither of these estrogens produced any action on bone marrow cells in tissue culture (groups *1A* and *1B* in Table III). The lack of action of estrogens in tissue culture is reviewed by Simonnet (10) although Schlag and Boll (11) claim estrogens have antimitotic action on bone marrow cells in culture. Bone marrow removed from mice, pretreated with estradiol one day before the culture was prepared, and with chlorotrianisene three days before the culture was prepared, did not show an increase in the total number of cells. Girod (4) found no increase in the mitotic index of granulocytes after treatment with estradiol but he reported that the mitotic index of the erythrocytic series was notably lowered.

On this medium differentiation proceeded rapidly. Maximum basophilia of the cytoplasm of the cultured cells of groups I, *1A*, and *1B* occurred by the twelfth hour of culture and in culture of groups II and III by the eighth hour.

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TABLE I.—RED BLOOD COUNT[a]

Group	Day 1	Day 14	Day 15	Day 18
I				
Male control	8,398,000 ± 1,280,000	9,363,000 ±834,000	8,730,000 ±897,000	8,938,000 ±930,000
II				
Male chlorotrianisene	8,169,000 ±830 000	8,790,000 ±960,000	8,938,000 ±863,000	9,158,000 ±906,000
III				
Male estradiol	8,276,000 ±965,000	8,430,000 ±913,000	8,794,000 ±995,000	9,060,000 ±949,000
IV				
Female control	7,630,000 ±1,794,000	8,960,000 ±1,228,000	9,720,000 ±1,345,000	9,960,000 ±1,402,000
V				
Female chlorotrianisene	8,656,000 ±1,876,000	8,090,000 ±1,560,000	9,310,000 ±1,602,000	10,010,000 ±1,720,000
VI				
Female estradiol	8,260,000 ±1,232,000	8,413,000 ±1,106,000	9,230,000 ±1,121,000	9,340,000 ±1,106,000

[a] Average of ten mice, with standard deviation.

TABLE II.—WHITE BLOOD COUNT[a]

Group	Day 1	Day 14	Day 15	Day 18
I				
Male control	9,125 ± 1,641	7,175 ± 1,564	8,225 ± 1,784	10,495 ± 934
II				
Male chlorotrianisene	8,760 ± 1,465	9,430 ± 1,270	12,530 ± 1,201	11,064 ± 896
III				
Male estradiol	8,834 ± 1,060	9,400 ± 1,020	9,260 ± 1,900	10,423 ± 1,040
IV				
Female control	8,905 ± 1,060	9,151 ± 1,302	9,069 ± 1,220	10,640 ± 1,068
V				
Female chlorotrianisene	8,460 ± 1,260	8,960 ± 1,296	8,950 ± 1,390	11,730 ± 1,112
VI				
Female estradiol	8,760 ± 934	9,010 ± 1,060	10,631 ± 1,439	9,631 ± 1,140

[a] Average of ten mice, with standard deviation.

TABLE III.—TISSUE CULTURE OF BONE MARROW

Group	No. Cells per ml. of Medium × 10^6 — Hours in Culture				No. of Granulocytes per 100 Nucleated Cells	
	Initial	3	8	12	36	Initial	After Hours
I	1.20	1.35	1.36	1.28	1.10	10	7
I*A*	1.30	1.30	1.32	1.22	1.20	11	8
I*B*	1.40	1.30	1.28	1.20	1.10	9	9
II	1.25	1.20	1.29	1.31	1.30	9	13
III	1.31	1.20	1.28	1.30	1.30	10	12

TABLE IV.—DIFFERENTIAL LEUKOCYTE COUNTS[a]

Group	Day 1				Day 14				Day 15				Day 18			
	L[b]	E[b]	N[b]	M[b]	L	E	N	M	L	E	N	M	L	E	N	M
I	65	0.2	23	12	66	0.1	22	12	64	0.2	24	12	65	0.1	23	12
II	65	0.2	23	12	66	0.1	23	11	63	0.1	25	12	53	0.1	36	11
III	64	0.1	24	12	65	0.1	24	11	44	0.1	46	10	59	0.1	30	11

[a] Average of ten mice, 100 leukocytes counted per mouse. [b] L, lymphocytes; E, eosinophils; N, neutrophils; M, monocytes.

Cells of the erythrocytic series modified quickly in this culture medium and disintegrated. Intra- and extracellular hemoglobin crystals were observed.

The various stages of the modified cells of the granulocytic series could be identified.

The number of granulocytes increased more rapidly in the cultures prepared from mice pretreated with estrogens than in those from untreated mice (Table III).

Girod (3–5) has repeatedly reported that injection of estradiol produced neutrophilia followed by neutropenia and that it increased the myelocytic line in bone marrow of treated animals.

CONCLUSIONS

Strain 101 male mice are more constant in red and white blood cell counts than female mice of the same strain.

Mild neutrophilia was produced by administering estradiol and chlorotrianisene.

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Improved Bioassay for Acetylcholine

By RALPH W. MORRIS

The pentobarbitalized, neostigminized dog carotid blood pressure preparation is proposed as a self-contained bioassay for acetylcholine that is not sensitive to concomitant drugs or ions which may be present. The dog blood pressure preparation has been found to possess certain advantages (e. g., reproducibility, stability, self-containment, simplicity, speed, etc.) over the classical preparations. Carotid blood pressure is recorded in the standard laboratory manner (i. e., carotid arterial cannula, mercury manometer, and kymograph recording). The statistical methods employed and the bioassay requirements satisfied are those stated by Burn in "Biological Standardization" (1).

RECENT EMPHASIS on the physiologic role of acetylcholine in the function of the central nervous system has renewed concern over certain difficulties with the classical bioassay preparations for acetylcholine. Such major difficulties as poor reproducibility, usually due to excessive variability between and within test preparations; lack of relevance, frequently the result of the presence of choline esters other than acetylcholine; and dissimilarity of solutions due to the presence of concomitant drugs or ions presented obstacles to the study of the effects of neuropharmacologic agents on the biosynthesis of acetylcholine. In order to circumvent these difficulties with the classical bioassay preparations, a modification of the cat blood pressure preparation of Brown and Feldberg (2) was developed. In this paper are described studies performed to establish the pentobarbitalized, neostigminized dog carotid blood pressure preparation as an improved bioassay preparation for acetylcholine.

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Grateful appreciation is extended to Drs. F. W. Schueler and H. H. Keasling for their assistance with the statistical portions of this paper.

EXPERIMENTAL

Procedure—Brown and Feldberg (2) gave few details of the procedures used to set up the cat blood pressure preparation; therefore, due to an inadequate supply of cats but a satisfactory number of dogs, the dog carotid blood pressure preparation was developed using routine laboratory procedures. For these studies of the dog blood pressure preparation, thirty-three healthy female mongrel dogs were anesthetized with pentobarbital sodium, 36 mg. per Kg. intravenously, prepared for recording of carotid blood pressure by a mercury manometer on a smoked kymograph, and sensitized to acetylcholine with neostigmine methyl sulfate, 60 mcg. per Kg. intravenously. The trachea was cannulated to facilitate respiration and a catheter inserted into the femoral vein through which all solutions were administered.

Calculations.—Brown and Feldberg (2) gave no statistical data on which the cat blood pressure bioassay preparation was based, therefore the bioassay and statistical methods of Burn and Finney (1) have been applied to the dog carotid blood pressure preparation. Depressor responses in the range of 15 to 40% were utilized in a four-point bioassay technique essentially that of Burn (1). The four-point technique assumes the selection of volumes of control and of experimental samples that will produce responses with magnitudes that match those obtained by a high and by a low concentration of a standard acetylcholine chloride solution. Since the acetylcholine dose-response curve is linear with a log dose-response relationship, the successively higher concentrations of the samples were always chosen as a constant multiple of the concentration of the next lower dilution (in this case, a multiple of two was used). All samples were assayed in duplicate or triplicate, utilizing a randomized block design.

The magnitude of the depressor response to acetylcholine was taken as the difference between the mean blood pressure before the response and the mean blood pressure at the peak of the depressor response. This difference was then transformed into per cent fall of the mean preresponse blood pressure by utilizing Eq. 1.

$$\frac{\bar{X}_{pre} - \bar{X}_{peak}}{\bar{X}_{pre}} \times 100 = \% \text{ fall of } \bar{X}_{pre} \quad \text{(Eq. 1)}$$

where $\bar{X}_{pre}$ = mean preresponse blood pressure and $\bar{X}_{peak}$ = mean peak response blood pressure.

The statistical methods of Finney (1) for relative potency, variance of M, fiducial limits, and probability were used on the transformed percentage data.

DISCUSSION

The requisites for any bioassay, as stated by Burn (1), are relevance, similarity, validity, linearity, and precision. In addition to these necessary features, a good bioassay should be rapid, self-contained, simple to conduct and to evaluate, and also possess a wide spectrum of responses although the slopes of the regression lines be steep (i. e., the sensitivity of the preparation should be adjustable).

Relevance, an important feature in any bioassay, is that the substance in question should be the only substance in the samples capable of eliciting the specific physiologic response to be quantitated (i. e., the acetylcholine induced vasodepressor response). To meet this requirement with the dog blood pressure preparation it was necessary to decrease the concentrations of adenosine triphosphate and choline chloride, two of the media constituents necessary for the biosynthesis of acetylcholine by brain tissues. Such a decrease eliminated all depressor responses arising from media constituents regardless of the volumes of solutions assayed while not greatly affecting the biosynthesis of acetylcholine.

Table I presents data from a typical bioassay after having performed the per cent transformation. Transforming the responses from mm. mercury fall of mean blood pressure to per cent fall of the mean preresponse blood pressure facilitated the comparison of data obtained from different dog preparations, as well as avoided the errors due to a changing preresponse blood pressure.

TABLE I.—BIOASSAY DATA AFTER PER CENT TRANSFORMATION[a]

Samples / Replicates	Standard Low Dose	Standard High Dose	Experimental Low Dose	Experimental High Dose	Control Low Dose	Control High Dose
IA	21.5	32.2	18.7	28.0	20.0	28.2
IB	26.6	34.1	21.4	27.9	18.2	28.0

[a] Per cent fall of mean preresponse blood pressure.

Table II is an analysis of variance of part of the data contained in Table I and is characteristic of the analyses performed on all the bioassay data. The one degree of freedom assigned to doses enabled a test for the significance of the differences between the combined high doses and the combined low doses of the two samples. The hypothesis that the potency of the experimental sample was correctly guessed (i. e., relative to the potency of the standard sample) was rejected when the probability for the one degree of freedom for doses was less than 0.05. The significant variance ratio for doses indicates that the doses of the experimental sample were misguessed (i. e., the doses chosen for the experimental sample gave responses that were comparable but significantly lower than those selected for the standard sample). Since there was no evidence of lack of parallelism, the misguessed potency does not invalidate the bioassay. If evidence of lack of parallelism had also been obtained, then the entire bioassay would have had to be repeated with emphasis on the proper matching of doses. The significant variance ratio for common regression is a good indication of regression of response on log dose. Data from all thirty-three preparations, as well as from this particular preparation, gave no evidence of a significant difference between replicates, therefore minimizing the importance of replicate assays. Nevertheless, duplicate assays were conducted as routine procedure.

TABLE II.—ANALYSIS OF VARIANCE OF DATA IN TABLE I

Source of variation	DF	Standard *versus* Experimental: Sums of Squares	Mean Squares	Variance Ratio (F)
Doses	1	42.32	42.32	18.32[a]
Common regression	1	144.50	144.50	62.55[b]
Lack of parallelism	1	0.72	0.72	0.31
Among samples	3	187.54	...	...
Between replicates	1	11.52	11.52	4.99
Within samples (error)	3	6.94	2.31	...
Total	7	206.00	...	...

[a] Significant ($P<0.05$). [b] Significant ($P<0.01$).

Another major requirement of a bioassay is similarity of solutions in that no solution contain any substance, other than the one to be assayed (i. e., acetylcholine) which might potentiate, inhibit, or otherwise modify the physiologic response to the test substance. Relevance and similarity differ in that relevance refers only to compounds capable of mimicking the standard acetylcholine depressor response (i. e., primarily choline esters such as butyryl, propionyl, or benzoylcholine) whereas similarity of solutions refers to the presence of drugs and/or ions in the test sample but not in the standard sample which might modify the acetylcholine induced vasodepressor response (i. e., potassium, calcium, choline, adenosine triphosphate, eserine, morphine, pentobarbital, etc.). As noted above, the media concentrations of adenosine triphosphate and choline chloride were reduced to eliminate all depressor responses arising from the media constituents. All other media ingredients such as potassium, magnesium, calcium, fluoride, cysteine, coenzyme A, phosphate buffer, and the drugs pentobarbital, phenobarbital, pentothal, morphine, levorphan, dextrorphan, nalorphine, levallorphan, dextrallorphan, reserpine, chlorpromazine, hydroxyzine, azacyclonol, and acetanilide did not alter the acetylcholine-induced vasodepressor response of the dog blood pressure preparation. This is in contrast to literature

reports of direct effects of diphenylhydantoin, phenobarbital, pentobarbital, picrotoxin, pentamethylene tetrazol, strychnine, camphor, scilliroside, and morphine on the frog rectus abdominis muscle preparation (3–5) and phenobarbital and diethyl barbiturate on the *Venus mercenaria* heart preparation (6); currently the two most frequently used bioassay preparations for acetylcholine.

Table III illustrates the typical dose-response data before and after two hours of acetylcholine assaying in which period of time drug accumulation, desensitization, and/or degeneration of the preparation could have occurred. Were these effects on the acetylcholine-induced vasodepression to have occurred, evidence of lack of parallelism would normally have appeared in the analysis of variance. There was no evidence of lack of parallelism in any of the thirty-three bioassays conducted, therefore indicating that these factors did not produce a significant effect on the dog blood pressure preparation.

TABLE III.—DOSE-RESPONSE BEFORE AND AFTER TWO HOURS OF ASSAYING

Samples	Standard		Experimental		Control	
	0.014 mcg. per Kg.	0.028 mcg. per Kg.	0.10 ml. Total	0.20 ml. Total	0.10 ml. Total	0.20 ml. Total
Replicates						
1, 0 hr.	20.3[a]	29.1	20.3	25.0	18.1	25.0
1, 2 hr.	...	...	19.3	26.1	19.4	21.9
2, 0 hr.	19.4	28.5	22.0	24.0	23.0	25.2
2, 2 hr.	...	...	20.8	24.0	15.8	26.6

[a] Per cent fall of mean preresponse blood pressure.

The four-point bioassay technique does not permit differentiation of deviations from parallelism into deviations due to nonlinearity and those due to differences in regression coefficients. A six-point technique would make such a differentiation possible. Since evidence of lack of parallelism of dose-response curves would invalidate a bioassay, each of the thirty-three preparations were investigated for lack of parallelism and, as stated above, no evidence of such noted. Therefore no need arose for using a six-point technique.

Validity is best demonstrated by obtaining identical potency calculations for a standard acetylcholine solution from the dog blood pressure bioassay preparation and from a second standard bioassay preparation. Spot checks of standard acetylcholine chloride solutions on the frog rectus abdominis muscle and the dog blood pressure preparations produced similar potency determinations, thereby validating the dog blood pressure preparation for the bioassaying of acetylcholine. No comparisons of biosynthesized acetylcholine were possible due to the presence in the media of concomitant ions and drugs which interfered with the response of the frog rectus abdominis muscle preparation (3–6).

Table IV shows the mean absolute sensitivities of the dog carotid blood pressure preparation and the classical bioassay preparations for acetylcholine. The sensitivity of the dog blood pressure preparation was increased seventeenfold following the administration of neostigmine methyl sulfate (mean total dose of 63.3 ± 6.5 mcg. neostigmine methyl sulfate per dog). Intermediate sensitivities could be achieved by decreasing the amount of neostigmine administered. By such regulation of the neostigmine dose the sensitivity spectrum of the dog blood pressure preparation could be adjusted so as to bracket the concentration range of the particular acetylcholine solution to be assayed, while retaining the characteristic steep slopes of the regression lines of the acetylcholine dose-response curves.

TABLE IV.—RELATIVE SENSITIVITIES OF BIOASSAYS FOR ACETYLCHOLINE

Assay	Mean mcg. Acetylcholine Chloride per ml. Test Solution
Hydroxamic acid (chemical assay)	4.50[a]
Frog rectus abdominis	1.50[a]
Frog rectus abdominis plus acetone	1.0×10^{-2}[a]
Frog rectus abdominis plus eserine	1.0×10^{-2}[a]
Frog heart	1.0×10^{-2}[b]
Leech longitudinal muscle	2.0×10^{-2}[b]
Cat blood pressure	1.0×10^{-2}[b]
Dog blood pressure	7.4×10^{-4}[a]
Venus mercenaria heart	5.0×10^{-4}[b]
Dog blood pressure plus neostigmine	4.3×10^{-4}[a]
Venus mercenaria heart plus eserine	5.0×10^{-6}[b]
Frog lung	Less than 10^{-10}[b]

[a] Data from this laboratory. [b] As cited in Gerard (7).

The precision of the dog blood pressure preparation is reflected in the variance of *M*: the logarithm of the relative potency of the test samples *versus* the standard sample. The arithmetic mean of thirty-three "variance of *M*" calculations is 36.2 ± 3.9% of the relative potency, *R*. This is comparable to the precision found in most bioassays.

The dog blood pressure preparation has several additional advantages over the classical preparations in that the rapidity of the individual responses (mean of 2.32 ± 0.09 minutes per response) enables a large number of responses to be performed on each preparation (mean of 115.2 ± 5.2 responses per dog). Second, the preparation could be utilized for several assays extending over a period of hours and occasionally even ten hours (mean of 270.0 ± 11.9 minutes per dog). Third, the rapidity and the large number of responses plus the stability and the duration of sensitivity of the preparation provide for a completely self-contained bioassay. Four, the dog blood pressure bioassay procedure is simple to perform, once the initial operation and setting-up have been completed.

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Interaction of Parabens with Nonionic Macromolecules

By SEYMOUR M. BLAUG and SAYED S. AHSAN

Methyl, ethyl, propyl, and butyl parabens interacted with various nonionic macromolecules frequently found in cosmetic and pharmaceutical formulations. In general, the binding tendency which the parabens exhibited for the various macromolecules increased with the molecular weight of the paraben. Thus, the strongest complexing tendency was exhibited by butylparaben, followed by propylparaben > ethylparaben > methylparaben. The hydrophile-lipophile balance of the macromolecules strongly influenced the binding tendency which the parabens exhibited for them. The parabens interacted to a greater extent with polyoxyethylene ester type macromolecules such as Tween 80 and Myrj 52 than with hydrophilic macromolecules like polyethylene glycols 4000 and 6000 and polyethylene polypropylene glycol.

THE INHIBITORY EFFECT of various nonionic macromolecules on the preservative action of many compounds, particularly the parabens, has been reported (1—7). This inhibitory effect may be due to the reported (8) interaction between the hydrogen of the phenolic hydroxy group in certain pereservatives with the basic oxygen in the ether groups of the polyoxyethylene chain found in many nonionic surfactants. Reports of similar interactions between pharmaceuticals and nonionic surfactants appear in the literature (9–11).

It was the purpose of this investigation to study the interactions of a series of *p*-hydroxybenzoic acid esters (methyl, ethyl, propyl, and butyl) with various nonionic macromolecules in order to determine the effect of increasing paraben chain length on the extent of interaction between the esters and the macromolecules. The macromolecules included in the study varied from strongly hydrophilic agents such as polyethylene glycols 4000 and 6000[1] to those containing hydrophobic moieties such as Tween 80[2] and Myrj 52.[3] The interaction between methyl and propyl parabens and Tween 80, Myrj 52, and polyethylene glycols 4000 and 6000 have previously been reported (8, 10, 11).

EXPERIMENTAL

The interaction of methyl, ethyl, propyl, and butyl parabens with Tween 80, Myrj 52, Pluronic F-68,[4] and polyethylene glycols 4000 and 6000 was studied according to the solubility method previously described (12). If complexing takes place to an appreciable degree, one observes either an increase or a decrease in the solubility of the paraben depending on the nature of the complex formed.

In Figs. 1–5 the solubility data was plotted to show the ratio, R, of total paraben in solution to the concentration of free paraben as a function of the macromolecule concentration. The R value can be used to approximate the total paraben concentration which must be employed in a system containing a known concentration of nonionic macromolecule assuming that any inhibition of paraben activity is due to interaction with the macromolecule. The R value multiplied by the required paraben concentration gives the total paraben concentration which must be employed (7).

Because of the variation in initial solubilities of the parabens, the relative complexing activity taking place in these systems can best be compared by comparing the per cent increase in solubilities of the parabens at a definite concentration of macromolecule.

PROCEDURE

Reagents.—Recrystallized methyl *p*-hydroxybenzoate, m. p. 127°; ethyl *p*-hydroxybenzoate, m. p. 118°; propyl *p*-hydroxybenzoate, m. p. 95–97°; butyl *p*-hydroxybenzoate, m. p. 73°; Tween 80; Myrj 52; Pluronic F-68; and polyethylene glycols 4000 and 6000.

Solubility Method.—Excess quantities of the parabens were accurately weighed and placed in 125-ml. glass-stoppered bottles together with varying quantities of the macromolecules. Sufficient water was added to each bottle to bring the volume to 50 ml. The bottles were placed in a mechanical shaker in a constant temperature bath and equilibrated at 25 ± 0.1° or 27°. Methyl and ethyl parabens were equilibrated for twenty-four hours and propyl and butyl parabens were equilibrated for forty hours. Ethyl and propyl parabens were equilibrated with Tween 80 and butylparaben was equilibrated with Tween 80 and Myrj 52 at 0° for forty-eight hours using an ice chest fixed to a mechanical shaker. After equilibration, aliquot portions of the supernatant liquid were removed and determined spectrophotometrically at a wavelength of 256 mμ since all of the parabens investigated showed a maximum absorb ance at this wavelength. A blank for each macromolecule concentration was used in order to compensate for any absorption by the macromolecule.

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[1] Products of Union Carbide Chemicals Co., New York, N. Y.

[2] Polyoxyethylene sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

[3] Polyoxyethylene stearate, Atlas Powder Co., Wilmington, Del.

[4] Polyethylene polypropylene glycol, Wyandotte Chemicals Corp., Wyandotte, Mich.

The quantity of each paraben used was as follows: methyl *p*-hydroxybenzoate 0.4 Gm.; ethyl *p*-hydroxybenzoate 0.4 Gm.; propyl *p*-hydroxybenzoate, 0.4 Gm.; and butyl *p*-hydroxybenzoate 0.8 Gm. Table I shows the effects of the various macromolecules on the solubility of each paraben in water.

DISCUSSION

From the data presented in Figs. 1–5 and in Table I it is evident that the parabens, especially propyl and butyl parabens, interact to a considerable extent with the polyoxyethylene ester type macromolecules. It can be seen that in general the binding tendency which the parabens exhibit for the various macromolecules increases with the molecular weight of the paraben. In other words, the strongest complexing tendency is exhibited by butylparaben, followed by propylparaben > ethylparaben >

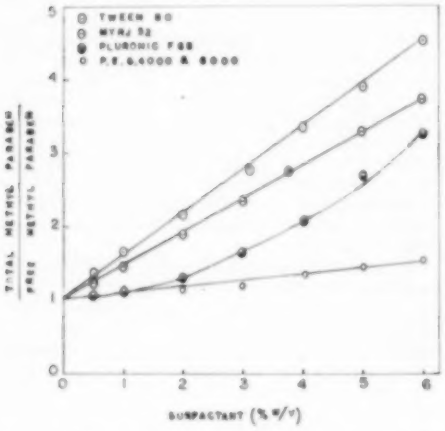

Fig. 1.—Binding of methylparaben by nonionic macromolecules in water at 27°.

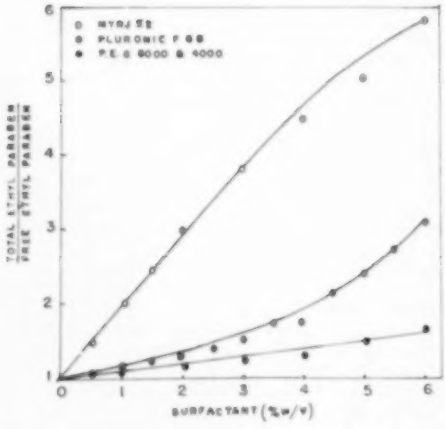

Fig. 2.—Binding of ethylparaben by nonionic macromolecules in water at 27°.

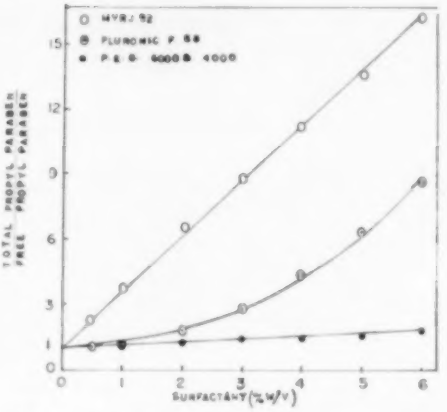

Fig. 3.—Binding of propylparaben by nonionic macromolecules in water at 27°.

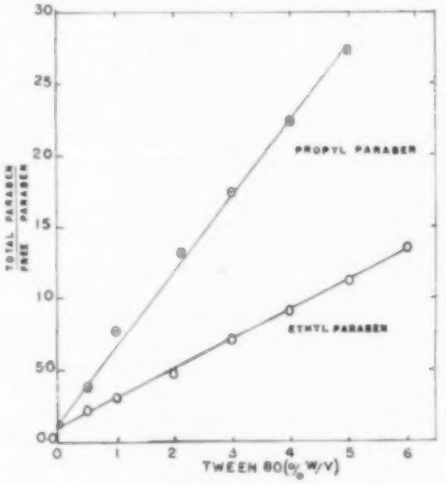

Fig. 4.—Binding of ethyl and propylparabens by Tween 80 in water at 0°.

methylparaben. The sharp increase in the solubility of butylparaben at the higher Pluronic concentrations may be due to micellar solubilization.

As indicated in Table I several of the interactions were studied at 0°. This was due to the formation of cloudy colloidal solutions at 25 or 27° which was probably due to the low cloud point temperature of the complexes formed between Tween 80 and ethyl, propyl, and butyl parabens and between Myrj 52 and butylparaben (13).

The binding tendency which the parabens exhibit for the macromolecules is strongly influenced by the hydrophile-lipophile balance of the macromolecule. The extent of complex formation in solutions of strongly hydrophilic macromolecules such as polyethylene glycols 4000 and 6000 is relatively very small when compared to the binding affinity ex-

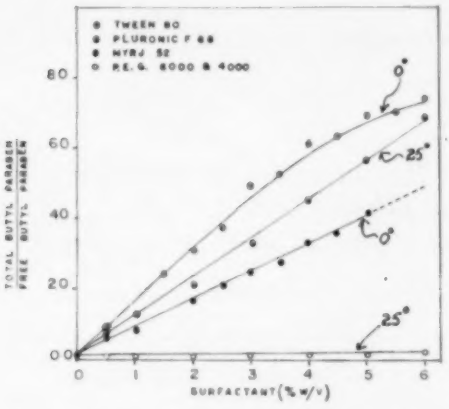

Fig. 5.—Binding of butylparaben by nonionic macromolecules in water at 0° and 25°.

TABLE I.—INTERACTION OF VARIOUS PARABENS WITH NONIONIC MACROMOLECULES AT 27°

Compound	Initial Solubility M/L × 10^{-2}	Increase in Solubility in 1% Solutions of Macromolecules, %				
		Myrj 52	Pluronic F-68	Tween 80	P.E.G. 4000	P.E.G. 6000
Methylparaben	1.60	146	107	158	107	108
Ethylparaben	0.95	199	114	310[a]	104	114
Propylparaben	0.23	364	115	781[a]	108	114
Butylparaben	0.12	851[a]	1000[b]	696[a]	113[b]	116[b]

[a] Solubilities determined at 0°. [b] Solubilities determined at 25°.

hibited by the polyoxyethylene macromolecules, Tween 80 and Myrj 52. The presence of hydrophilic, hydrophobic moieties in the interacting molecules renders the replacement of water molecules hydrogen bonded to the basic ether oxygen of the polyethers, more favorable (14).

If we assume that the preservative activity of the parabens is due to the presence of free or unbound paraben then the *R* values shown in Figs. 1–5 should enable one to calculate the amount of paraben required for any macromolecule concentration up to 5%. For example, in Fig. 1 the *R* value for methylparaben in the presence of 5% Tween 80 is 4. This indicates that if the concentration of free or unbound methylparaben required to inhibit the growth of microorganisms is 100 mg./100 ml. in the absence of Tween 80 it would be four times that or 400 mg./100 ml. in the presence of 5% Tween 80 in order to insure the presence of 100 mg./100 ml. of unbound methylparaben. The correlation of binding data with preservative concentration has been demonstrated *in vitro* by Pisano and Kostenbauder (7) for methyl and propyl parabens in the presence of Tween 80. However, in a study conducted by Charles and Carter (15) on the effect of sorbic acid and methyl and propyl parabens on organism growth in typical nonionic emulsified commercial cosmetics, it was shown that these preservatives provided adequate protection against organism growth in preparations inoculated with various organisms. The *p*-hydroxybenzoate esters were more effective in finished formulations than one would predict from the binding data. This was probably due to the change from the simple aqueous system in which the binding studies were conducted to the complex cosmetic emulsion system. The preservation of cosmetic and pharmaceutical emulsions undoubtedly depends upon more factors than interaction between preservative and nonionic macromolecule. However, consideration of such interactions in formulation work should not be overlooked. If binding data are available one can at least obtain an approximation of the required preservative concentration when a nonionic macromolecule is included in a formulation.

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Application of C-Reactive Protein Responses in the Evaluation of Acute Drug Toxicity

By RICHARD F. RILEY, MONROE COLEMAN, Y. HOKAMA, and WILLIAM HANAFEE

C-REACTIVE protein is an abnormal serum protein with the electrophoretic mobility of a β-globulin which is not present in normal serum but appears therein as a result of inflammation (1). While it is commonly found in the blood during the acute phase of various inflammatory diseases, it can also be elicited experimentally in twenty-four to thirty-six hours by administration of a wide variety of noxious organic and inorganic agents (2).

Provided a supply of specific antiserum, one can easily follow the appearance and disappearance of this substance. Antiserum is commercially available for human C-reactive protein; while antiserum against the analogous rabbit protein, Cx-reactive protein is not commercially available, it can be prepared with a little effort (3).

It occurred to us that the use of this response might provide a rather quick, simple method of screening the tissue compatability of various drugs and vehicles. While it would not supplant such recognized methods as histological examination of tissues involved, evaluation of effect of topical application on the cornea, etc., it should supply supplemental information of value in predicting tissue tolerance to various parenteral routes of administration of therapeutic doses of potentially irritating drugs and vehicles. Since pharmacologists do not appear to be generally aware of this response, the purpose of this note is to provide two comparative examples of this response in support of the view set forth above. The first compares the Cx-protein response of rabbits to subcutaneous injection of two vehicles of types used as menstrua for drugs to be injected subcutaneously or intramuscularly. The second example compares the C-reactive protein response to intravenous and intra-arterial injection of 50% sodium diatrizoate as employed clinically by the diagnostic radiologist in intravenous pyelography and cerebral angiography. In both examples the acute phase responses seem to support the subjective impression gained in regard to comparative tolerance to these procedures.

EXPERIMENTAL

Determination of Cx- and C-Reactive Proteins.—Antiserum to Cx-reactive protein was obtained by injecting subcutaneously Cx-protein-Cx-polysaccharide complexes (4) mixed in adjuvant into the inguinal region of guinea pigs weighing 500–600 Gm. The animals were bled during the third or fourth week postinjection. Antiserum to C-reactive protein was obtained commercially from Schieffelin and Co., New York. Both Cx- and C-reactive protein levels were determined semiquantitatively by a capillary precipitation method (5). The acute phase protein titers were taken as the highest dilution of the serum giving a visible precipitate with the specific antiserum.

Serum for test was obtained from blood collected from the marginal ear veins of rabbits and by venipuncture from clinic patients.

Stimulation of Cx-Protein by Injection of Vehicles.—Two vehicles were tested. The first consisted of 1% carboxymethylcellulose dissolved in physiological saline, and the second consisted of an emulsion of equal parts of medicinal mineral oil, physiological saline, and the ointment base, Aquaphor. The emulsion was injected subcutaneously in doses of 3–4 ml. in the nape of the neck in a group of 15 rabbits. Twelve of the 15 rabbits gave maximum Cx-protein titers of 1:8 or 1:16 twenty-four hours after the injection. The remaining three gave responses of 1:1, 1:2, and 1:4. All were initially negative. A group of five rabbits were similarly injected with 10 mg. of carboxymethylcellulose in 4 cc. of saline. Cx-protein titers twenty-four hours after injection were negative in four of five rabbits, and the fifth had a titer of 1:1.

Stimulation of C-Protein in Response to Intra-arterial and Intravenous Administration of Sodium Diatrizoate.—Blood samples were drawn for C-protein test by venipuncture from in-patients at the time they were examined in the diagnostic section of the Radiology Department of the U.C.L.A. Medical Center. There were two groups consisting of 10 patients who were examined by cerebral angiography and 10 patients who received intravenous pyelograms. During the course of the roentgenographic examinations, those patients examined by cerebral angiography received at least two rapid intra-arterial injections of 10–12 ml. each of a 50% solution of sodium diatrizoate into one or both carotid arteries. The total dose generally ranged from 40 to 48 ml. Patients examined by intravenous pyelography received 30 ml. of the same drug by slow infusion into the antecubital vein. Repeat blood samples for C-protein test were obtained twenty-four hours after the roentgenological examinations.

The effect of these procedures on the C-protein response is shown in Table I and Table II.

A number of these patients had levels of C-protein prior to examination as a result of inflammation attendant on the indicated pathology. Nevertheless, it is clear that an inflammatory response was

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TABLE I.—C-REACTIVE PROTEIN TITERS OF SERUM OF INDIVIDUALS BEFORE AND TWENTY-FOUR HOURS AFTER INTRAVENOUS PYELOGRAMS

Diagnosis	Pretiter	Post-titer
Hyperthyroidism	1:8	1:16
Tabes	1:1	1:1
Diabetes	Neg.	Neg.
Prostatic hypertrophy	1:32	1:16
Diverticulitis	1:64	1:128
Peripheral vascular disease	Neg.	Neg.
Fever, undetermined origin	1:32	1:64
Duodenal ulcer	1:8	1:8
Neurogenic bladder	1:8	1:8
Pyelonephritis	1:128	1:256

TABLE II.—C-REACTIVE PROTEIN TITERS OF SERUM OF INDIVIDUALS BEFORE AND TWENTY-FOUR HOURS AFTER CEREBRAL ANGIOGRAPHY

Diagnosis	Pretiter	Post-titer
Cholestoma	1:1	1:8
Temporal lobe cyst	1:4	1:8
Chronic lung infection	1:64	1:64
Epilepsy	Neg.	1:4
No disease	Neg.	1:8
Cerebral arteriosclerosis	1:1	1:16
Carcinoma of rectum	1:1	1:8
Ideopathic epilepsy	1:1	1:16
Brain tumor	Neg.	1:4
No disease	Neg.	1:1

initiated or increased more markedly in those patients who received the drug by fast intra-arterial injection than by slow intravenous infusion. Thus, in none of the patients receiving the drug by slow intravenous infusion did the C-protein titer increase more than one dilution. In the patients receiving the drug by fast intra-arterial injection the level was raised four dilutions in three of them and three dilutions in four instances. This is not surprising in view of the hypertonic nature of the drug employed; thus while the osmotic pressure of serum is about 7.6 atm. at 37° we calculate that of a 50% solution of hypaque sodium to be about 40 atm. at 37°, assuming complete dissociation. The much more frequent incidence of acute toxic manifestations seen clinically following cerebral angiography than after intravenous pyelography provides very strong subjective evidence of the more irritating nature of the former procedure.

SUMMARY AND DISCUSSION

The observations presented above together with a number scattered throughout the fairly extensive clinical literature dealing with C-reactive protein responses, suggests that tissue-compatable drugs and mild surgical procedures do not elicit a C-reactive protein response. Conversely, absence of a response to an unstudied agent or procedure would imply that it produced less than some minimum amount of tissue damage.

Quantitative stimulus-C-reactive protein response data in man or experimental animals are not presently available, however, C-reactive protein responses in various clinical situations suggests that the intensity of the response correlates with the subjective clinical impression of the intensity of the stimulus (6). Preliminary data we have obtained for rabbits indicates that Cx-reactive protein responses will correlate with the intensity of the stimulus and that a threshold intensity of stimuli can be found below which responses will not occur.

Experimentally elicited Cx-protein responses, then, appear to offer a measurable end point which could be utilized in designing bioassays of some interest. For example, it should be possible to interrelate the intensity of diverse stimuli in terms of their respective magnitudes to produce some selected mean level of C-reactive protein response.

Further, we have found that the Cx-reactive protein response in the rabbit to relatively mild stimuli can be blocked by simultaneous administration of fluorometholone in doses which are incapable of preventing responses after more intense stimuli (7). This observation suggests that a quantitative comparison of inciting stimuli can be carried out in terms of a blocking dose$_{50}$ of an agent such as fluorometholone, the drug dose being defined in terms of a selected standard stimulus. Conversely, the response might be employed in comparative assays of anti-inflammatory drugs using a standard stimulus for Cx-reactive protein. These possibilities are being explored.

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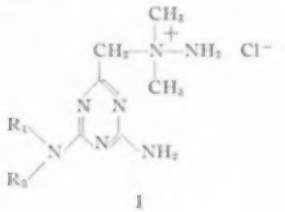
1-(Substituted Triazinylmethyl)-1,1-dimethylhydrazinium Chlorides. Guanamines IX

By SEYMOUR L. SHAPIRO, ELAINE S. ISAACS, and LOUIS FREEDMAN

Condensation of chloromethylguanamines with dimethylhydrazine gave hydrazinium chlorides (I), some of which have muscle relaxant activity.

CONDENSATION of a variety of chloromethylguanamines (1) with dimethylhydrazine gave hydrazinium chlorides, I, (2, 3).

Compounds having structural features related to those in this series (4) show a broad spectrum of pharmacological effects, and examination of I to this point has indicated muscle relaxant activity particularly with compounds 1 and 2.



I

General Procedure for the Preparation of 1-[(2-Amino-4-substituted Amino-1,3,5-triazin-6-yl)-methyl]-1,1-dimethylhydrazinium Chlorides.—The halomethylguanamine (1) (0.01 mole) dissolved in 40 ml. of acetonitrile was treated with 0.6 Gm. (0.01 mole) of dimethylhydrazine in 30 ml. acetonitrile. After several hours, the formed precipitate was recrystallized to give the products described in Table I.

TABLE I.—HYDRAZINIUM CHLORIDES (I)

No.	R_1	R_2	M. p., °C.[a]	Yield, %[b]	RS[c]	Formula	Analyses, %[d] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	n-C_4H_9-	H	190–191	39	A	$C_{10}H_{22}ClN_7$	43.6	44.0	8.0	7.9	35.6	35.7
2	n-C_5H_{11}-	H	182–184	33	A	$C_{11}H_{24}ClN_7$	45.6	45.7	8.4	8.3	33.8	34.4
3	i-C_5H_{11}-	H	173–176	37	A	$C_{11}H_{24}ClN_7$	..	..	..	..	33.8	34.4
4	-$(CH_2)_5$-		211–212	58	A	$C_{11}H_{22}ClN_7$	..	..	..	..	34.1	33.8
5	$C_6H_5CH_2$-	CH_2-	120–121	42	B	$C_{14}H_{22}ClN_7$	..	..	..	..	30.3	30.6
6	p-$CH_3C_6H_4$-	H	224–225	39	C	$C_{13}H_{20}ClN_7$	50.4	50.4	6.5	6.6	..	..
7	o-$CH_3C_6H_4$-	C_2H_5-	213–214	37	A	$C_{15}H_{24}ClN_7$	53.3	52.8	7.2	7.0	29.0	29.3
8	p-ClC_6H_4-	H	242–243	17	D	$C_{12}H_{17}Cl_2N_7$	43.6	44.0	5.2	5.5	..	..
9	p-$CH_3OC_6H_4$-	H	229–230	33	A	$C_{13}H_{20}ClN_7O$	47.9	48.4	6.2	6.3	..	..

[a] Melting points are not corrected and were established on a Fisher-Johns melting point block. [b] Yields are reported as recrystallized product. [c] RS is recrystallizing solvent: A = ethanol-hexane; B = isopropyl alcohol-hexane; C = methanol-ether; D = ethanol. [d] Analyses are by Weiler and Strauss, Oxford, England.

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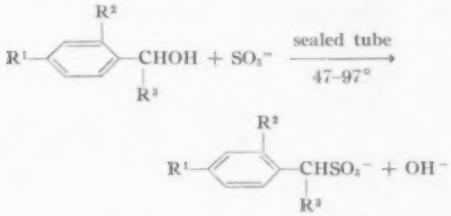
Preparation of Hydroxybenzylsulfonic Acids

By LOUIS C. SCHROETER† and TAKERU HIGUCHI

COMPOUNDS fitting the following model were found to react rapidly with bisulfite or sulfite yielding benzylsulfonates:



where either R^1 or R^2 must be —OH; R^3 may be —H, $—CH_2$, $—CH_2R^4$ (R^4 may be various amino, amido, or alkyl radicals) (1). At somewhat higher temperatures (130°) in sealed tubes, *p*-alkoxybenzyl alcohols likewise undergo the reaction (2, 3). Higher reaction temperatures required for these compounds apparently reflect differences in dipole moment and resonance effect between hydroxy- and alkoxy-benzyl alcohols. The reaction appears to follow a typical Sn_2 mechanism; second-order kinetics have been observed at pH values between 5 and 8 (4).

Preparative value of this general reaction between bisulfite and hydroxybenzyl alcohol model compounds may be enhanced by simply heating the reactant solution under reflux in a nitrogen atmosphere (5). It is the purpose of this communication to describe briefly a general synthetic procedure using salicyl alcohol and epinephrine as models. Similar yields may be obtained with other reactive compounds using slight procedural modifications. When the product is a zwitterionic amino sulfonate, isolation is considerably simplified inasmuch as these compounds usually precipitate from concentrated reaction mixtures, thus obviating the need for the ethanolic extraction step.

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EXPERIMENTAL

Equimolar amounts (0.05 mole) of the substituted benzyl alcohol and sodium bisulfite or sulfite and about 200 ml. of water were placed into a 3-necked flask fitted with a reflux condenser, dropping funnel, and a tube for flushing the solution with nitrogen. The solution was refluxed for eight hours while keeping it flushed with nitrogen to prevent sulfite oxidation. Standard hydrochloric acid (1 *N*) was added periodically to maintain pH range 6 to 7. After refluxing, the solution was cooled to 25°. Unreacted starting material was removed by extracting with an immiscible solvent. Evaporation of the neutral aqueous solution gave a mixture of benzylsulfonate and inorganic salts. Extraction of the residue with hot ethanol yielded the desired product which was then purified by repeated recrystallizations from ethanol-water.

Sodium Hydroxyphenylmethane Sulfonate (from 2-Hydroxybenzyl Alcohol).—Yield, 6.95 Gm. (66%).

Anal.—Calcd. for $C_7H_7NaO_6S$: C, 40.00; H, 3.36; Na, 10.94; S, 15.26. Found: C, 39.29; H, 3.38; Na, 10.89; S, 14.96.

1-(3,4-Dihydroxyphenyl)-2-methylaminoethyl Sulfonate (from Epinephrine).—Yield, 10.8 Gm. (87%).

Anal.—Calcd. for $C_9H_{13}NO_5S$: C, 43.71; H, 5.29; N, 5.66; S, 12.97. Found: C, 43.65; H, 5.23; N, 5.24; S, 12.96.

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Synthesis and Pharmacological Activities of Some N-(2,2,3-Trichloro-1-hydroxypropyl)-amides

By JOSEPH P. LaROCCA

The amides of 2,2,3-trichloropropionaldehyde were synthesized and their physical and chemical constants determined. These compounds showed significant anticonvulsant activity, N-(2,2,3-trichloro-1-hydroxypropyl)-acetamide being the most active.

PREVIOUS WORK in this laboratory has shown the potentialities of chlorinated amides as sedative-hypnotics and anticonvulsants (1, 2). The method of synthesis described by LaRocca, *et al.*(3), with slight modification, was employed to prepare a series of N-(2,2,3-trichloro-1-hydroxypropyl)-amides. Nine of these compounds have been prepared (Table I). In addition, N-(2,2,3-trichloro-1-hydroxypropyl)-2-pyrrolidone was prepared by the same method (m.p. 129°[1]; yield 85.5 per cent. Analysis for nitrogen[2]—Calcd.: 5.68. Found: 5.69, 5.72). These compounds were screened for pharmacological activity.

TABLE I.—N-(2,2,3-TRICHLORO-1-HYDROXYPROPYL)-AMIDES ($RCONHCH(OH)CCl_2CH_2Cl$)

Amide	Yield	M. P., °C.[a]	Nitrogen[b] Calcd.	Nitrogen[b] Found
Acetamide	82.8	168	6.38	6.30 6.39
Chloroacetamide	84.3	155	5.49	5.56 5.57
n-Butyramide	98.4	132	5.63	5.68 5.70
iso-Butyramide	77.6	136	5.63	5.66 5.61
n-Valeramide	91.6	124	5.33	5.17 5.20
iso-Valeramide	81.0	108	5.33	5.67 5.80
Benzamide	87.9	135	4.96	4.61 4.64
Phenylacetamide	93.9	124	4.72	4.31 4.44
Nicotinamide	90.8	155	9.88	9.62 9.68

[a] All melting points on calibrated Fisher-Johns apparatus.
[b] Microanlyses by Spang Microanalytical Laboratory, Ann Arbor, Mich.

RESULTS OF PRELIMINARY TESTING

Toxicity.—Preliminary tests indicated that all of the compounds are relatively nontoxic. The approximate LD_{50} i. v. for mice is 100 mg./Kg. Mice fed a diet containing 0.5% of the acetamide derivative (approx. 300 mg./Kg./day), for a period of thirty days showed no apparent ill effects.

Sedative-Hypnotic Activity.—With these compounds, doses of 100 mg./Kg. intraperitoneally in rats do not produce hypnosis. As the dosage levels approach 200 mg./Kg., the animals are very depressed and most are prostrate. The hypnotic activity shown by doses of 200 mg./Kg. is approximately that demonstrated by pentobarbital sodium in doses of 20 mg./Kg.

Anticonvulsant Activity.—N-(2,2,3-Trichloro-1-hydroxypropyl)-acetamide, in oral doses of 300 mg./Kg., protects rats and mice for a period of seven hours against hourly doses of 20 mg./Kg. of metrazol given i. v. It is interesting to note that diphenylhydantoin will protect mice against metrazol convulsions, but not rats.

Conditioned Response.—When compared with reserpine for effect on conditioned response, by a standard testing procedure, these compounds were found to be inactive.

Analgesic Activity.—None of these compounds exhibited any analgesic activity.

EXPERIMENTAL

Materials.—2,2,3-Trichloropropionaldehyde, furnished by the Union Carbide Chemicals Co.

Synthesis.—Equimolar portions of 2,2,3-trichloropropionaldehyde and the appropriate amide were mixed and stirred. In most cases the reaction mixture solidified readily without the necessity of heating. If solidification did not occur within thirty minutes, the reaction was then heated on a steam bath for from three to four hours. Isolation and purification of the product was accomplished in the manner previously described (3). All the compounds are white crystalline solids, insoluble in water, but soluble in alcohol and acetone.

SUMMARY

1. Ten new derivatives of 2,2,3-trichloropropionaldehyde have been synthesized and characterized.

2. Pharmacological data shows these compounds have significant antiepileptic activity. Of this series, N-(2,2,3-trichloro-1-hydroxypropyl)-acetamide is currently undergoing clinical evaluation for possible antiepileptic use.

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Pharmacological results are made possible through the courtesy of Jack Keith, Research Pharmacologist, S. E. Massengill Co.
Presented to The Scientific Section, A. PH. A., Washington, D. C., meeting, August, 1960.
[1] All melting points on calibrated Fisher-Johns apparatus.
[2] Microanalyses by Spang Microanalytical Laboratory, Ann Arbor, Mich.

Determination of Vitamin A

Criticism of Tardif's Method

Sir:

Selective destruction of vitamin A as a basis of spectrophotometric estimation has a long history, mainly associated with irradiation techniques; see, for instance, Little (1). For the same purpose Tardif (2) has recently suggested the use of a nonselective destroyer, sulfuric acid, with the vitamin preparation dissolved in hexane. However, in attempting to take account of the effect of the agent on the ultraviolet absorption of the foreign material in the preparation, Tardif proposes a mathematical formula whose rationality is unclear.

Algebraically, the problem is that we have four unknown quantities (absorption at 325 mμ):

	Initially	After Partial Destruction
Vitamin A	A	a
Accompanying foreign material	F	f

and it is required to find the value of A, given the following three quantities: observed absorption of the preparation; initially, $M_1 = A + F$; after partial destruction, $M_2 = a + f$. Destructive effect on pure vitamin A as determined on a standard sample, $R = a/A$.

Obviously, without supplementary information, the problem is insoluble, for there are more unknowns than equations. The "solution" given, but not explained, by Tardif (see his Eq. 3) is

$$A = \frac{M_1^2 (1 + R^2)}{M_1^2 + M_2^2}$$

This yields $A = M_1$ when $R = M_2/M_1$; in other words, whenever the destruction is proportionately the same for both the vitamin and the foreign material, the formula obligingly indicates that no foreign material was present in the original preparation. And in other situations, comparably spurious results will emerge.

Tardif does not give a worked example, so we shall have to resort to hypothetical situations by way of illustration. The following three sets of data (compositions in arbitrary spectrophotometric units) are typical enough:

		I	II	III
Initially	A	85	90	95
	F	15	10	5
After partial destruction	a	5.37	5.68	6.00
	f	1.63	1.32	1.00

Now in all three cases the analyst would observe $M_1 = 100$, $M_2 = 7$, and $R = 0.063$. So he would infer that the three preparations were identical in composition and, applying the prescribed formula, he would find that $A = 99.9$ (i. e., 0.1 per cent irrelevant absorption) for each, whereas in fact, all A's are different and the largest is only 95 (i. e., all have at least 5% irrelevant absorption).

Herein, too, lies the explanation of the good reproducibility of results reported by Tardif. Clearly, if all results tend to an artificial closeness to a maximum, an apparently high precision will be observed. But precision, apparent or otherwise, is of scant value if accuracy is wanting and, unless these two concepts are lucidly distinguished, statistical analysis of results is fraught with dangers.

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Determination of Vitamin A

Rebuttal to Gridgeman Criticism

Sir:

Reminding the reader that this proposed correction (Eq. 3) cannot safely be used when $R = M^2/M$ is a constructive criticism, since this theoretical mathematical limitation was omitted in the original paper. The expectation that spurious results will emerge, however, is unrealistic and may be caused by a lack of understanding of the experimental behavior of the saponification, the extraction of the unsaponifiables by a selective solvent, and the destruction procedure.

To resort to hypothetical situations as a means of checking the validity of this correction is an acceptable routine. But one should always be sure that the conditions chosen are those prevailing during experimentation, otherwise the conclusions are fraught with danger.

Experimental results obtained with a Canadian Reference Standard and with a sample (blank preparation + Vitamin A Standard) are given in Tables I and II.

TABLE I.—CANADIAN REFERENCE STANDARD CAPSULE (25,000 I. U./CAPSULE)

	Absorbance[a], mμ		
Wavelengths	310	325	334
A	0.440	0.518	0.452
a	0.002	0.007	0.009
$R = a/A$	...	0.0135	...
A (Eq. 4)	...	0.517	...
Potency	...	24,170 ± 621	...

[a] After saponification, extraction, and dilution to 10 I. U./ml.

TABLE II.—SAMPLE (125 ML. BLANK + 1 STANDARD CAPSULE)

	Absorbance[a], mμ		
	310	325	334
$M_1 = A + F$	0.465	0.518	0.450
$M_2 = a + f$	0.047	0.030	0.033
$R_1 = (a + f)/(A + F)$	...	0.058	...
A (Eq. 4)	...	0.457	...
A (Eq. 3)	...	0.517	...
Claim, % (Eq. 4)	...	85.5	...
Claim, % (Eq. 3)	...	96.5	...

[a] After saponification, extraction, and dilution to 10 I. U./ml.

Table III shows what would happen if the sample of Table II had a vitamin A content equal to 85 or 115% of its potency. In working out the absorbance values for these two hypothetical situations, consideration must be given to the vitamin A and foreign material behavior: the A absorbance is proportionately changed while the F absorbance remains unchanged.

TABLE III.—HYPOTHETICAL SITUATIONS FROM EXPERIMENTAL DATA OF TABLE II

	Experimental Data	Hypothetical Situations—85% Vitamin A	Hypothetical Situations—115% Vitamin A
M_1 or $A + F$	0.518	0.440	0.596
A	0.517	0.439	0.595
F	0.001	0.001	0.001
M_2 or $a + f$	0.030	0.029	0.031
a	0.007	0.006	0.008
f	0.023	0.023	0.023
R_1 or M_2/M_1	0.058	0.066	0.052
A (Eq. 3)	0.516	0.438	0.595

Table IV shows a realistic correction of Gridgeman's hypothetical situations.

TABLE IV.—REALISTIC CORRECTION OF GRIDGEMAN'S HYPOTHETICAL SITUATIONS[a]

Initially	A	(85)	(90)	(95)
	F	5[b]	5[b]	(5)
		(15)	(10)	
After partial destruction	a	(5.37)	(5.68)	(6.00)
	f	15.00[b,c]	15.00[b,c]	15.00[b,c]
		(1.63)	(1.32)	(1.00)
R_1 or M_2/M_1		0.226	0.218	0.21
		(0.07)	(0.07)	(0.07)
A (Eq. 3)		86	91	96

[a] Numbers in parentheses are values selected by Gridgeman.
[b] F and f values do not change when the vitamin A level is raised or lowered, providing the initial dilution is kept.
[c] Absorbance f is always at least three to five times greater than F.

The A values calculated with Eq. 3 are all in good agreement with their respective experimental or hypothetical values (Tables II, III, and IV).

Gridgeman's explanation (artificial closeness to a maximum) for this high degree of accuracy cannot be accepted: the true vitamin A potency was repeatedly obtained from all samples investigated. Is this not the object of the proposed correction?

REAL TARDIF

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